


2006

Characterization of the Rank Ligand Positive Giant Cell Found in the Interfacial Membrane

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**CHARACTERIZATION OF THE RANK LIGAND POSITIVE GIANT CELL
FOUND IN THE INTERFACIAL MEMBRANE**

A thesis submitted in partial fulfillment of the requirements for the degree of Master of
Science at Virginia Commonwealth University

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May, 2006

ACKNOWLEDGEMENT

I would like to thank my committee members Dr. Jack Haar and Dr. Roger Loria for their enthusiasm and advice. My sincere thanks and appreciation goes to my advisor Dr. Matthew Beckman, whose patience, knowledge and encouragement made this all possible.

Thanks to the members of my lab for their humor and ability to make this past year a very enjoyable one. A special thanks to Sam Ramage for being a tireless source of guidance and aid throughout this research project.

Thank you to Kimberly Haydu for being by my side through all the challenges of the past year.

I would not be where I am today without the constant love and support of my parents, my brother, and the rest of my family. They give me strength and drive at every turn of the road, may they always be as proud of me as I am of them.

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LIST OF ABBREVIATIONS

IFM	interfacial membrane
LPS	lipopolysaccharide
M-CSF	macrophage-colony stimulating factor
MW	molecular weight
NF- κ B	nuclear factor-kappa B
OCIF	osteoclast inhibition factor
OCT	optimal cutting temperature
OPG	osteoprotegrin
PBS	phosphate buffered saline
RANK	receptor activator of nuclear factor kappa B
RANKL	receptor activator of nuclear factor kappa B ligand
RT-PCR	reverse transcriptase-polymerase chain reaction
TACE	Tumor necrosis factor converting enzyme
THA	total hip arthroplasty
TJA	total joint arthroplasty
TKA	total knee arthroplasty
TNF α	tumor necrosis factor alpha
TRAF	TNF receptor associated factor
TRAP	tartrate resistant acid phosphatase
IL-1 β	Interleukin 1 beta
IL-6	Interleukin 6
RPGC	RANKL positive giant cell
CMV	Cytomegalovirus
VDR	Vitamin D receptor
CTR	Calcitonin receptor

ABSTRACT**CHARACTERIZATION OF THE RANK LIGAND POSITIVE GIANT CELL
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By Patrick Jones, B.S.

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of
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Virginia Commonwealth University, 2006

Director: Dr. Matthew J. Beckman

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Aseptic osteolysis is a major complication to total joint arthroplasty requiring several thousand people a year to have to undergo revisions of their joint prosthesis. The formation of the interfacial membrane has been associated with aseptic osteolysis leading to the failure of all types of total joints. Recent evidence suggests that RANKL, a potent activator of bone reabsorption, is present in the interfacial membrane. Prior research in this laboratory to determine the source of RANKL in the interfacial membrane has revealed the presence of intense areas of RANKL concentration in the membrane. These areas of RANKL concentration correspond to multiple nuclei and a distinct cellular structure in the tissue as determined through light microscopy. This structure either represents a multi-nucleated giant cell or a cluster of cells that express high concentrations of RANKL. These following studies attempted to characterize this cell and determine its lineage.

The results of these studies show that the RANKL producing anomaly appears multi-nucleated in all examples with a RANKL staining pattern that makes it appear as a multi-nucleated cell. Furthermore this RANKL positive giant cell (RPGC) stains negative for markers typically seen on myeloid cells, osteoclasts, and osteoblasts. This RPGC does however stain very well for fibroblast markers and the inflammatory cytokines TNF- α , IL-1 β , and IL-6. The most interesting result from these studies revealed that this cell was positive for cytomegalovirus and expressed high concentrations of TNF- α converting enzyme (TACE). These data does lead to a hypothesis as to how this cell might form and how large an impact it might play in the interfacial membrane with respect to aseptic bone reabsorption.

GENERAL INTRODUCTION

Aseptic Osteolysis

There are approximately 1.5 million joint replacement surgeries performed every year and total joint replacement is widely regarded as one of the most successful surgical procedures in medicine today [1]. However a condition known as aseptic osteolysis has become a major complication to the long term outcome of these joint replacement surgeries. According to a retrospective study conducted between 1996 and 2003, of 439 revision hip surgeries done nearly 70% percent of them were due to periprosthetic osteolysis leading to aseptic loosening [2]. The process of periprosthetic osteolysis is defined as the progressive destruction of periprosthetic bony tissue, characterized on serial radiographs as progressive radiolucent lines and cavitations at the implant of cement-bone interface [3]. This disease take place in the absence of bacterial infection as can be diagnosed by pre-operative synovial fluid cultures and post operative tissue culture. The destruction of the bone during aseptic osteolysis is due to chronic bone reabsorption by osteoclasts until fixation of the implant with the bone is lost [4]. Once thought to be a biological response to wear particulate or a particulate disease [5], it has been shown that this is too simplistic an explanation as there is a wide variety of implant material used, yet it has been shown that wear particles do play an important role [6]. The dominant theory today is that wear particles stimulate cellular responses which initiate osteolysis through chemical signaling [7]. The wear particles most often associated with the disease are titanium and polyethylene debris generated from mechanical friction in

the joint [8, 9]. Polyethylene particles between the sizes of 0.1 and 1.0 micron have been shown to be the most reactive [10].

Osteolysis can be classified as either linear which presents radiographically as a small generalized linear pattern or aggressive where it presents as large erosive patterns. Osteolysis has been characterized in knee and hip arthroplasty as a cause for revision surgery.

Osteolysis is characterized by the formation of the interfacial membrane (IFM) around the bone cement interface. This membrane has histological and histochemical characteristics of a synovial-like lining. The synovial-like cells are oriented adjacent to the cement layer, while a deeper layer of macrophages forms in closer proximity to the bone face. T cells are for the most part absent or comparatively few in numbers [11]. There is no significant difference between the IFM of cementless and cemented joints. The cementless joints show more fibroblastic connective tissue though these observations are not significant. The IFM does stain strongly for IL-1 and TNF-alpha in membranes from cementless and cemented. The cell participants of the disease include macrophages, osteoblasts, fibroblasts, and osteoclasts. However, as was shown by Jiranek et al., macrophages and fibroblasts were the dominant cell types and less than 10% of the cells were T lymphocytes [12]. The relative absence of lymphocytes and the variation in material used in arthroplasty give evidence to the fact that osteolysis is not a lymphocyte driven immune response against any component of the prosthesis. Rather osteolysis is a more general disease involving inflammation and changes in cell signaling. The presence of RANKL has been detected in the interfacial membrane in many recent studies and may lead to a better explanation of what cells are mediating aseptic osteolysis [13].

RANKL/RANK/ OPG signaling pathway

The ligand of Receptor activator of NF- κ B which is more commonly known as RANK Ligand plays a significant role in aseptic osteolysis. RANKL is a 317 amino-acid long protein that is a member of the TNF/TNF receptor super family. The primary function of RANKL is the stimulation of osteoclastic differentiation from osteoclastic precursors. Additionally RANKL will activate osteoclasts and provide an anti-apoptotic signal to mature osteoclasts [14]. The primary target of RANKL is the receptor RANK on pre-osteoclastic cells. The interaction of RANKL and RANK will stimulate the expression of genes required for osteoclastic differentiation [15]. The role of RANKL and NF- κ B in the formation of osteoclasts has been demonstrated through murine models and pharmacological blockade. Childs et al. showed that in a mouse model with an antibody block of RANK-RANKL signaling there was a loss of wear debris-induced osteolysis [16]. Additionally, Kong et al. demonstrated that RANKL knock-out mice showed severe osteopetrosis and a systemic depletion of osteoclasts [17]. Two years later in 2004 Clohisy showed that a pharmacological blockade of NF- κ B signaling inhibits particle stimulated osteolysis [18]. Recently, Sabokar et al. showed that when RANKL and sufficient m-CSF were added to a culture of periprosthetic membrane derived macrophages that formation of osteoclasts would result. Additionally Sabokbar showed that this formation of osteoclasts could be inhibited by osteoprotegrin (OPG) when it was added to the culture [19]. RANKL has been shown to have a membrane bound form as described above but also a soluble form. The soluble form of RANKL is produced by the interaction of membrane bound RANKL and TNF converting enzyme (TACE). TACE acts like scissors and clips RANKL from the membrane where it is being expressed and

releases it into extra-cellular environment. The soluble form of RANKL is still capable of binding to RANK and mediating its effects [20]. RANKL is typically produced by osteoblasts and stromal cells in response to parathyroid hormone and other hormonal and inflammatory mediators [21]. However recently it has been shown that membrane derived fibroblasts are capable of producing RANKL and supporting osteoclastogenesis when properly stimulated. The presence of RANKL producing fibroblasts has been linked to stimulation with titanium wear particles as was shown by Wei et al. [22]. Additionally, human osteoblasts have been shown to produce RANKL in response to polyethylene particles [9]. Titanium and polyethylene represent two of the major materials used in the production of joint implants.

The receptor of RANKL is RANK which stands for receptor activator of NF- κ B. RANK mediates the pro-osteoclastic differentiation signal to pre-osteoclastic cells. RANK is a member of the TNF receptor family and it mediates its effects when bound by RANKL which is also called TRANCE, ODF, or OPGL. RANK is a transmembrane protein that is 616 amino acids long. RANK is typically expressed on cells of myeloid lineage thus including monocytes, macrophages, dendritic cells, and lymphocytes. Many of these types of cells are also pre-osteoclastic cells and have the ability to differentiate into osteoclasts when given the proper signaling [14]. In the case of osteoclastic differentiation the proper signaling involves cell to cell interaction of RANKL expressing cells with RANK expressing cells. RANK signaling in pre-osteoclastic cells involves the binding of the cytoplasmic RANK domains with TRAF-2, 5 and 6 [23]. Osteoclast precursor cells demonstrate an interaction of TRAF-6 and cytoplasmic RANK domains when extracellular RANK domains are bound by RANKL [15]. The effect of TRAF-6 in

the RANK signaling pathway can be further demonstrated by the fact that TRAF-6 deficient mice show symptoms of osteopetrosis and have improperly functioning mature osteoclasts [24].

The last part of the RANKL/RANK signaling pathway is osteoprotegrin (OPG). OPG is produced by mesenchymal cells that are also associated with RANKL production however OPG lacks a transmembrane region and thus is secreted from the cells producing it into the extracellular fluid [25]. OPG like RANK has several names; it is also known as osteoclast inhibition factor (OCIF) or TNF receptor like molecule 1 (TR1). The function of OPG is to act as a soluble decoy receptor of RANKL [26]. To this effect, the binding of OPG to RANKL effectively blocks RANKL's ability to bind RANK and cause osteoclastic differentiation. The name osteoprotegrin translates to "protector of bone". OPG is a protein that is synthesized in the cell as a protein of 401 amino acids but is later cleaved into a 380 amino acid molecule when it is secreted [14]. OPG is classified as a heparin binding glycoprotein with seven structural domains that is part of the TNF receptor superfamily however unlike many other members of that family as stated above OPG is not membrane bound, it is secreted by stromal cells and osteoblasts which are the same cells that produce RANKL [27]. The importance of OPG inhibition of RANKL signaling has been demonstrated in that OPG knockout mice showed severe osteoporosis which was ultimately the result of increased bone reabsorption [28].

The role of m-CSF has been demonstrated by Takami et al. They showed that in order for RANK/RANKL interaction to cause osteoclastogenesis there must be sufficient m-CSF present. However, m-CSF was shown not to be necessary to activate mature osteoclasts to begin reabsorbing bone as RANKL is [29]. Thus, it can be concluded that while m-

CSF is a necessary co-factor of RANK/RANKL mediated osteoclastogenesis it is not necessary for RANKL/RANK mediated activation of mature osteoclasts or the inhibition of osteoclasts apoptosis.

TNF- α , IL-1 β and IL-6

The wear particulate generated through mechanical grinding of the bone against the prosthesis following joint arthroplasty leads to inflammation in the joint. This inflammation is mediated by pro-inflammatory cytokines. Work by Jiranek et al. (1993) determined that though there are many different cytokines in the IFM the most prevalent ones based on immunostaining were IL- and TNF-alpha [12]. IL-6 has also been associated with the interfacial membrane and seems to be produced by fibroblasts in the membrane [30, 31].

TNF-alpha is a homotrimer of 157 amino-acid subunits first cloned in 1984. TNF-alpha is primarily produced by activated macrophages and mediates many pro-inflammatory effects. TNF-alpha signals through the surface receptors TNF-R1 and TNF-R2. Of these two receptors it has been determined that TNF-R1 mediates the majority of TNF-alpha's biological effects. TNF-alpha's signaling pathway eventually results in the activation of the transcription factor NF- κ B. To this effect, TNF- α signals in the same method as RANK and explains why they are in the same family of receptors. NF- κ B activation occurs through the phosphorylation-dependent ubiquitination and degradation of I κ B proteins. NF- κ B is usually bound by this inhibitor protein however once they have been degraded NF- κ B is free to move into the nucleus from the cytoplasm and cause transcriptional change [32].

Interleukin 1 is one of the most important pro-inflammatory cytokines and there are three variants of it found in the body. IL-1 α , IL-1 β , and IL-1Ra are the three members of the IL-1 gene family. IL-1 α and beta are both synthesized as precursors without leader sequences. Following translation, they have a molecular weight of 31,000, however following processing by cellular proteases into their mature form they drop to a molecular weight of 17,000. IL-Ra is conversely translated with a signal peptide and is excreted from the cells prior to being modified by proteases. Similar to TNF- α , IL-1 β also leads to the activation of the inhibitory kappa B complex and the destruction of I κ B allowing NF- κ B to be activated and enter the nucleus. Of all the variants of IL-1, it is IL-1 β that appears to play the most significant role in osteolysis [33]. Macrophages isolated and cultured from the interfacial membrane are capable of producing large amounts of IL-1 β in response to wear particles typically found in the IFM [33]. Additionally, it has been seen that in patients with aseptic loosening of hip replacements there is a serum elevation of IL-1 β [34].

Moreschini et al., hypothesized that these two inflammatory cytokines of IL-1 β and TNF- α play a direct role in the formation of aseptic osteolysis through the stimulation of RANKL expression and thus increase osteoclastogenesis [35]. The question of how the two cytokines up-regulate RANKL is still unclear. Whereas Ma et al., demonstrated that IL-1 β is able to increase osteoclastogenesis in a murine model when there are permissive levels of RANKL. This process seems to be independent of TNF- α [36]. Additionally a more recent study has shown that TNF-induced RANKL up-regulation is actually mediated by IL-1 β . To this effect, TNF- α is unable to stimulate osteoclastogenesis on its own [37]. It was also demonstrated independently by two

groups that TNF- α alone cannot activate mature osteoclasts to begin reabsorbing bone [38, 39]. However, these two studies have been challenged in recent years as a study by Fuller et al., showed that TNF- α does potentially activate mature osteoclasts through a RANKL independent manner, but that it is synergistic with RANKL [40]. Furthermore, Sabokbar et al. demonstrated that TNF-alpha is sufficient to induce osteoclasts differentiation from arthroplasty macrophages provided that there is sufficient m-CSF present. The study by Sabokbar et al. also showed that TNF- α and IL-1 α were capable of stimulating bone reabsorption independently but that in culture they could act synergistically. Thus, there appears to be a RANKL/RANK independent system for stimulating osteoclastogenesis and osteoclasts activation. This would be likely to operate in a system such as the interfacial membrane where there is a high level of cytokine production due to the wear particulate being generated [41]. Whether TNF- α and IL-1 are able to stimulate osteoclastogenesis independent of RANKL, or whether they simply up-regulate RANKL, is still unclear.

IL-6 has been shown to be present in the membrane in high levels particularly in areas of dense fibroblasts as opposed to IL-1 and TNF- α which are more localized to the areas occupied by high numbers of macrophages [42]. It appears based on current research that IL-6 is produced by fibroblasts in the membrane in response to IL-1 and TNF- α [31]. IL-6 is produced from monocytes and T-cells in response to trauma in the body. IL-6 signals through the JAK-STAT pathway and is expressed when mechanical trauma or stress is placed on the body. The grinding of the prosthesis on the bone could thus be a stimulus for IL-6 production in aseptic osteolysis.

Recent evidence from the Cleveland clinic has suggested that bacterial endotoxin found on the prosthesis and on wear particulate can accumulate in the IFM. This accumulation of endotoxin is a stimulus for inflammatory cytokines to be released by macrophages and thus endotoxin would contribute to the over all environment of inflammation in the IFM [43].

Cell Composition of the IFM

Classical characterization of the IFM indicates it is composed primarily of fibroblasts and macrophages [12]. Macrophages are phagocytes of the innate immune system that are major producers of inflammatory cytokines and mediators of inflammation. Macrophages in the interfacial membrane have been shown to actively engulf wear particulate from the prosthesis which in turn causes them to release more cytokines [5]. Macrophages play a dual role in osteolysis in that they are the major cell type host defense responding to wear particles via ingestion and the production of cytokines $\text{TNF-}\alpha$ and $\text{IL-1}\beta$, however they also are the precursors for osteoclasts responsible for ensuing bone reabsorption [10]. The function of osteoclasts is the reabsorption of bone through enzymes that break down the collagen matrix and remove phosphate and calcium ions into the blood. An over abundance of osteoclastic bone reabsorption is the root problem in osteolysis.

Prior research in this laboratory

RANKL is a potent activator of bone reabsorption. The focus of osteolysis research for the past 4 years in this lab has been RANKL present in the membrane and what its source might be. This began in 2002 with a study by Beckman et al. where characterization of the IFM as having two distinct layers based on cellular composition.

The layer of the IFM closest to the bone was composed of significantly greater ($p < 0.001$) numbers of macrophages while the IFM layer closest to the prosthesis was composed of significantly greater ($p < 0.001$) numbers of fibroblasts (**Table 1**). The study also demonstrated that the two layers of IFM differ based on cytokines present. TNF- α gene expression was detected uniformly throughout the IFM, whether close to bone or close to the implant. In contrast, IL-1 β gene expression was much more significantly detected in the IFM adjacent to the bone, whereas, IL-6 gene expression levels were found to be significantly higher in the IFM near the prosthesis than near to bone [44]. During this study, RANKL was detected in RNA isolated from the interfacial membrane. RNA was isolated from the IFM in areas against lytic bone reabsorption versus from control non-lytic areas (**Figure 1**). This approach demonstrated that IFM tissue against areas of lytic bone reabsorption are significantly greater in the concentration of RANKL than in those areas of IFM that were forming against intact bone [45]. This evidence suggested that RANKL was present in the IFM and played a role in bone reabsorption. This was confirmed in a study by Li et al. (2006b) in which fibroblasts isolated from the IFM were positive for RANKL and negative for CD14 [46]. Observations of the IFM using RANKL fluorescent antibodies and 400x microscopy revealed a large RANKL positive cell (**Figure 2**). Upon further investigation of this cell, it was also noted that it stained negative for multiple macrophage markers and positive for fibroblast markers and appeared multi-nuclear[47]. The presence of a multi-nuclear fibroblast suggests several different mechanisms of cell fusion, one of those being viral involvement.

Table 1: Characteristics of different layers of interfacial membrane

Table 1A: Shows the observed cellular composition of IFM as determined through five field counts at 400x of tissue sections. (p <0.001)

Table 1B: Shows the cytokine profiles of the two different layers of the IFM. (p<0.001)
(tables courtesy of Mengnai Li)

A.

Cell Type	Bone Layer	Implant Layer	<i>p</i> Value
Macrophage	208±11	100±13	0.001
Fibroblast	43±41	160±13	0.001
Lymphocyte	3±2	2±2	0.755
Osteoclast	2±1	0	0.001

B.

Cytokine	Bone Layer	Implant Layer	<i>p</i> Value
IL-1	2.76±0.41	0.86±0.57	<0.001
IL-6	0.65±0.45	1.78±0.36	<0.001
TNF	2.76±0.71	2.53±0.60	0.853
PDGF	1.08±0.51	1.03±0.50	0.783

Figure 1: RANKL concentration in the IFM

Figure 1 shows the greatly elevated RANKL levels at the site directly associated with a lytic lesion as opposed to control IFM harvested from sites away from the lesion in the same respective patient ($p < 0.001$)
(figure courtesy of Mengnai Li)

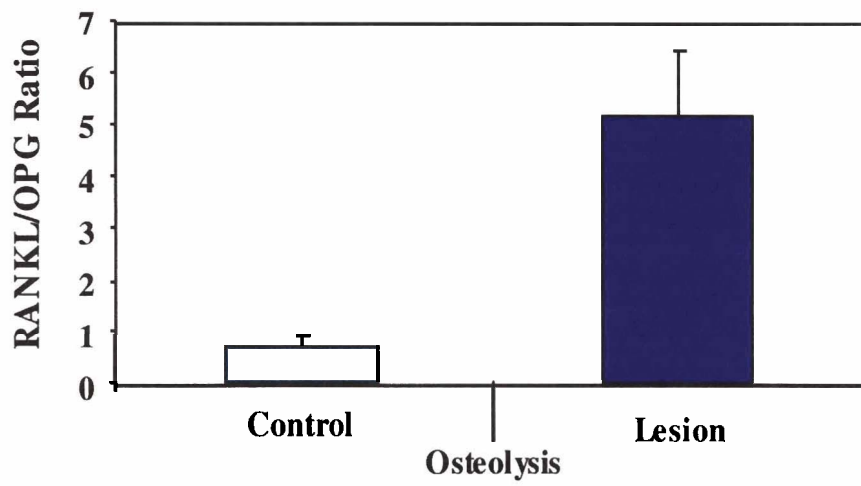


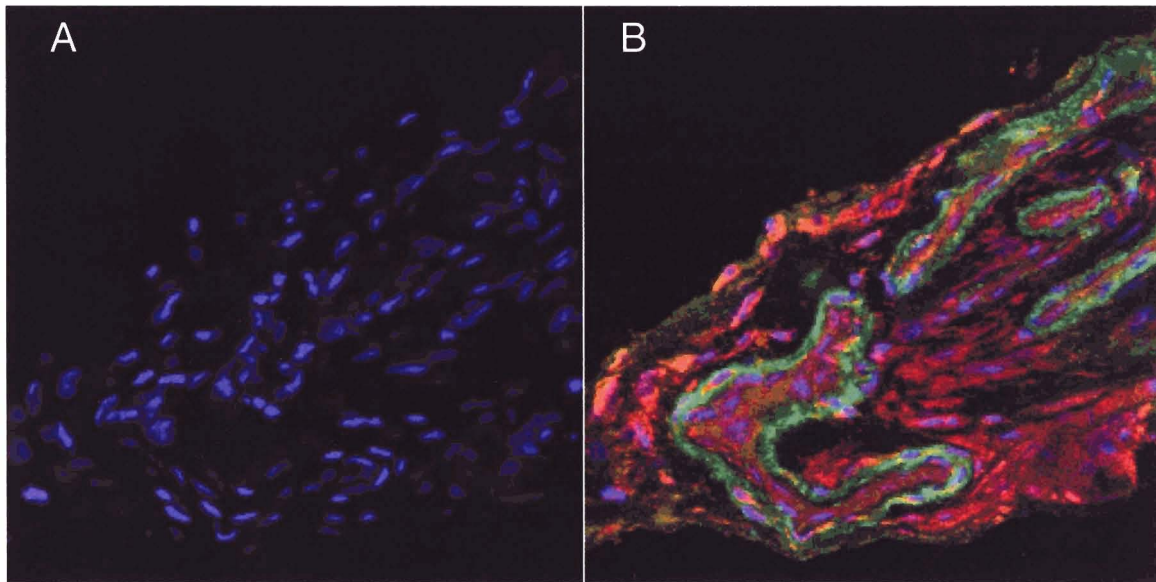
Figure 2: First observation of a RANKL positive giant cell in IFM

Figure 2A: Panel A shows an image (400x) of the nuclei (blue) associated with this image. Whereas, Panel B depicts a composite picture of antibody staining for 5B5 (red) and RANKL (green).

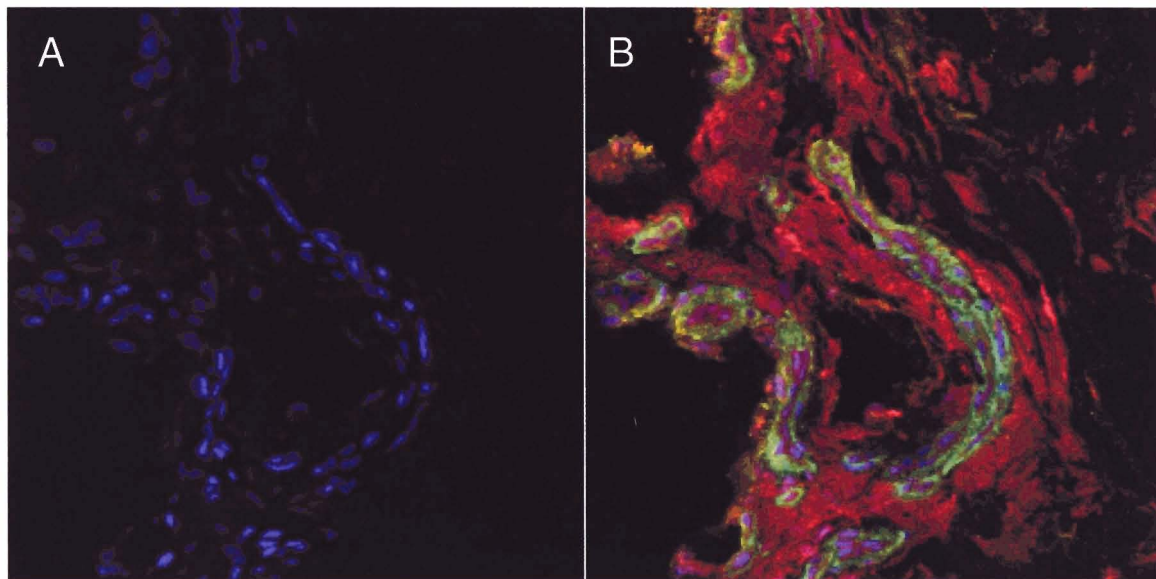
Figure 2B: Panel A shows an image (400x) of the nuclei (blue) associated with this image. Panel B shows the composite picture of RANKL (green) and BerMac3 (red) antibodies stains.

(images courtesy of Sam Ramage)

A.



B.



Viral Mediated Cell Fusion

Cells fused by virus are generally considered harmless and nonfunctional, however recent evidence suggests that these fused cells are capable of proliferation and survival and that their existence can mediate negative consequences on the body [48]. There are several viruses implicated in cell fusion of which a large group is the herpes family of viruses.

Cytomegalovirus is a member of the herpes family of viruses and shares the characteristic of most herpes like viruses in that once infected with CMV a patient will carry the virus for life and shed it intermittently [49]. CMV has the largest genetic content of the human herpes viruses with a DNA genome of 240 kbp. It is estimated that close to 90% of the population is infected with CMV however the symptoms are mild enough to escape diagnosis upon initial infection. It is also estimated that at any given time about 10% of the population is shedding CMV. It appears that the primary trigger for CMV to be brought out of latency is immunosuppression and for this reason it is a major cause of death in transplant recipients and AIDS patients. Infection can be detected histologically through observation of foci of swollen retractive cells with cytoplasmic granules. These cells are multi-nucleated and have smooth round acidophilic cytoplasmic masses, however a hallmark of infected cells are pathognomonic large amphophilic “skein like” nuclear inclusion bodies. CMV infected cells are typically swollen and are multi-nucleated in addition to having multiple perinuclear cytoplasmic inclusions and intranuclear inclusions. A study by Garnett demonstrates that CMV infection of fibroblasts causes the formation of giant multi-nucleated cells [50]. The absence of bacterial infection in aseptic osteolysis does not rule out the possibility of viral

involvement since assays to determine bacterial infection could easily miss viral infection.

Focus of current project

The major focus of the following research will be the characterization of the RANKL positive giant cell, which will be referred to as (RPGC). This RPGC was stained with an extensive battery of antibodies in order to clarify its lineage, role in the IFM tissue, and the possible role of CMV in its fusion. Furthermore all studies presented will involve an increase in imaging used on this cell from 400x to 630x. The increase in magnification will attempt to determine if this RPGC is in fact a multi-nucleated cell or a dense group of cells collectively expressing RANKL. We hypothesize that the RPGC in the IFM tissue is a multi-nucleated fibroblast-like cell characterized by intense RANKL expression and formed through fusion of fibroblasts that were infected by CMV.

CHAPTER 2

IMMUNOFLUORESCENT ANALYSIS OF THE RANKL PRODUCING GIANT CELL FOUND IN THE INTERFACIAL MEMBRANE (A CASE STUDY)

ABSTRACT

This case study was to attempt to characterize the RANKL expressing giant cell or cluster of cells seen in all interfacial membranes examined by this lab. This case study was performed on tissue taken from a 77 year old female undergoing revision hip surgery due to aseptic osteolysis. This patient displayed a rapid progression of osteolysis and a high level of the RANKL expressing cells. Tissue removed from this patient was frozen and cut into 12 micron sections. The sections were stained with antibodies to fibroblast cells (D7-fib), antibodies to macrophages (Ber-Mac), and antibodies to inflammatory cytokines (TNF- α , IL-1 β , and IL-6). In addition antibodies to cytomegalovirus were used in the cells characterization. For the purposes of this study all samples were incubated with RANKL antibody and its presence was used in identification of the cell to which the staining of the other antibodies was characterized.

The results showed that the RPGC stained negative with the Ber-Mac anti-macrophages antibody. The RPGC did however stain positive with the D7-fib anti-fibroblast antibody and all three inflammatory cytokines. The RPGC also demonstrated positive staining for the CMV antibody.

This study first documents the existence of a multi-nucleated cell in the interfacial membrane that expresses high concentrations of RANKL. Our data suggest that this RPGC is not of myeloid lineage, is positive for the presence of TNF- α , IL- β , IL-6 and is most likely fibroblast-like. In addition this RPGC is positive for CMV antigen.

INTRODUCTION

The formation of the interfacial membrane is a characteristic of periprosthetic osteolysis leading to total joint failure. This membrane forms between the prosthesis and the bone and has been referred to as synovial-like due to the thin layer of polygonal cells (1-2 cells thick) that forms abutting the cement or prosthesis[11, 51]. However away from the prosthesis this membrane becomes very thick and is composed primarily of fibroblasts and macrophages. In this sense, interfacial membrane (IFM) is perhaps a misnomer and should be referred to as a tissue, however, in this document the tissue and membrane will be labeled IFM or IFM tissue. The IFM has been uniquely associated with osteolysis in that when revisions of joint arthroplasty are done for reasons of mechanical failure or sepsis the IFM is not observed. The composition of the IFM tissue was determined in 1993 by Jiranek et al. as being primarily composed of fibroblasts and macrophages with relative low numbers of T cells and osteoblasts [12]. The RANKL/RANK/OPG system has been implicated in the development of osteolysis with some question given to the source of the RANKL. There have been recent reports demonstrating that RANKL is expressed in fibroblasts isolated from the IFM and that it is capable of inducing osteoclastogenesis in vitro that could mediate bone reabsorption [52, 53]. In previous studies using confocal microscopy and immunofluorescence in this lab, a large multi-nucleated cell or cluster of cells that stains very intensely with RANKL has been observed.

The purpose of this study was to characterize this cell and determine the cell type it might be using antibody staining and high power confocal microscopy. Although all IFM tissue samples observed have demonstrated examples of this multi-nuclear cell there

are very few of them per tissue section. One patient however showed a remarkably high amount of these cells in her tissue as is demonstrated in **Figure 3**. The relative abundance of these multi-nuclear cells in this periprosthetic tissue made the patient a great case study with which to further characterize this cell. To characterize the lineage of this cell, many serial sections were made of the tissue and stained with a panel of antibodies to determine the classification of this cell that so highly expresses RANKL. The antibodies used were specific for cells of mesenchymal or myeloid origin. This direction was based on the composition of the IFM as determined through prior work documenting that fibroblasts and macrophages were the two major cell types present in the IFM [42]. Additionally, the presence of inflammatory cytokines in the interfacial membrane has been well characterized with particularly high levels of TNF- α , IL-1 β and IL-6. Therefore, emphasis was also on relating the staining pattern of RANKL with the individual staining patterns of these three cytokines.

The patient chosen for this study showed a high level of the cell in question when observed using a 40x objective (**figure 3**). However for the purposes of cell characterization the power of the scope was increased to a 63x objective to allow more detail to be seen. The tissue used for this study was obtained from a 77 year old female undergoing revision of the acetabular component of a right hip prosthesis on 11/7/05. The patient received her primary hip arthroplasty on 2/11/00 at age 71. Thus the patient was able to tolerate the implant for a little over 5 years. However the patient was required to have a femoral component revision on 8/6/2000 due to fracture and sepsis and not the result of osteolysis. The patient's serial radiographs between October 2000 and May 2003 showed no signs of osteolysis and it was noted by the surgeon at the time of her May

2003 clinic visit that the patient exhibited “no lucency and good bone stock”. This was followed by the same surgeon’s preoperative notes in 2005 as stating that “the patient’s acetabulum is grossly loosened and sitting at essentially 90 degrees of abduction, the patient has a very large superolateral defect that would be graded as a type II”. The difference of approximately 2.5 years between these two radiographic reports suggests an extremely rapid form of osteolysis around the acetabular component. This case is of particular interest due to the fact that osteolysis was clearly observed as not being a factor in 2003 and less than 3 years later had led to complete loss of fixation of the acetabular component. The correlation between the patient’s rapid loss of fixation coupled with a high number of these RANKL expressing cells is worth noting.

MATERIALS AND METHODS

Tissue preparation

Tissue was obtained from one 77 year old female undergoing revision hip surgery. The interfacial membrane was kept at 4° C following removal from the patient; the tissue was then embedded in OCT compound (Tissue-Tek) and frozen at -20°C until it was sectioned. The sections were made at 12 microns on a cryostat and melted on to superfrost microscope slides (Fisher Scientific). Frozen sections were kept at -20°C until stained.

Antibody Staining

Frozen sections were fixed using ice cold 100% methanol for 60 seconds and then washed with PBS. The slides were blocked with a 3% goat serum PBS mixture and washed in .01% triton for ten minutes. The triton wash was to decrease non-specific binding. Antibodies were prepared in PBS dilutions with 1% goat serum. Primary antibodies were diluted 1:50 and secondary antibodies were diluted 1:200. Antibodies were incubated for 1 hour at room temperature and following each incubation the sections were washed 5 times with PBS and once with a .01% triton solution to minimize non-specific binding. All sections were stained with Hoechst 34580 stain diluted 1:200 in PBS. **Table 2** summarizes all information on antibodies used in this study.

Analysis of Immunolabeling

Slides were observed using a Carl Zeiss LSM 510 Meta confocal imaging microscope. The lasers used for analysis were the 405nm diode laser, a 488nm Argon laser, and a 543nm Helium Neon laser. The first image of the tissue to determine abundance or giant cells was taken using a 40x oil immersion objective. Individual cells

were observed using a 63x oil immersion objective. In each case the characteristic intense RANKL staining was used to target cells for analysis. The sections were analyzed using FITC fluorescence to locate the characteristic intense RANKL staining. Once located the examples were checked with light microscopy to ensure that a distinct cell was present. In all examples a Hoechst nuclear stain was used to determine if examples were multi-nucleated or not as this was a characteristic of previously examined cells.

Table 2: Antibodies used in case study

Table 1 summarizes the information on the antibodies used in this study including their name, host animal, fluorescent secondary and supplier.

antibody	primary host animal	Secondary antibody	Supplier
CMV monoclonal	mouse	543 chicken anti-mouse	Chemicon International
RANKL polyclonal	goat	488 donkey anti-goat	Santa Cruz Biotechnology
RANKL monoclonal	mouse	543 chicken anti-mouse	R & D systems
Mac3 monoclonal	mouse	543 chicken anti-mouse	Dako Corporation
TNF-alpha monoclonal	mouse	543 chicken anti-mouse	R & D systems
IL-1 monoclonal	mouse	543 chicken anti-mouse	R & D systems
IL-6 monoclonal	mouse	543 chicken anti-mouse	R & D systems
D7-fib monoclonal	mouse	543 chicken anti-mouse	Gene Tex

RESULTS

DIC imaging of the multi-nuclear giant cell

The use of the 63x objective allowed for increased magnification and greater resolution. This increase in magnification led to the ability to visualize the morphology of this giant cell under light microscopy. The light images were taken using a Differential Interference Contrast (DIC) filter on the confocal microscope where only the halogen lamp was ignited. The light images revealed a definitive structure in the interfacial membrane that corresponded to the RANKL staining. The DIC imaging gave validity to the RANKL image that the antibody was specifically binding to a structure as seen in **figure 4**. This characteristic structure was then used to evaluate all further images and confirm that in each case a defined cellular shape was observed in addition to the fluorescence. The multiple Hoechst stains inside the cell indicating multiple nuclei are a characteristic of this cell (**figure 4**). All examples observed were multi-nucleated.

Controls

In order to control for non-specific binding, several control slides were made and analyzed. The first set of controls consisted of secondary antibody controls. In that every primary antibody used in this study was not coupled to a fluorescent antibody it was necessary to use secondary fluorescent antibodies raised against the host species of the primary antibody. For this set of experiments the RANKL antibody used in all sections to target the giant cells was a polyclonal antibody raised in goats against humans. An anti-goat secondary antibody that was designed to excite when hit with a 488nm laser was used as its secondary. All other primary antibodies used in this study and which were evaluated for their co-localization with RANKL, were raised in mice against humans.

These antibodies were detected by an anti-mouse secondary-conjugated to a fluor group designed to excite when hit with a 543nm laser. When evaluated alone these secondary antibodies showed limited or no fluorescence indicating that they did not specifically bind to the tissue. Additionally, since polyclonal RANKL antibody was used in every section it was necessary to confirm that it was binding specifically to RANKL. To this effect, a section was stained with both RANKL polyclonal antibody and RANKL monoclonal antibody. The two antibodies bound to the same cell indicating that the RANKL polyclonal antibody was specifically binding to RANKL and validating the RANKL binding in other tissues.

Fibroblast and macrophage specific antibodies

The purpose of this study was to characterize this multi-nucleated cell. Based on previous research, it has been demonstrated that the interfacial membrane is composed primarily of fibroblasts and macrophages [42]. It was thus necessary to determine if this multi-nucleated cell was either of macrophage or fibroblast origin. To examine this, the tissue section was incubated with D7-fib a very specific monoclonal antibody to antigen on fibroblasts and epithelial cells. The staining of D7 was very specific for the RANKL producing cell and demonstrated considerable intensity in staining.

Mac3 is a very specific marker for cells of myeloid origin. The human form of it is also known as Ber-Mac3 and has been used in many studies involving the IFM to identify macrophages[54]. The staining with Ber-Mac3 demonstrated no co-localization with the RANKL producing cell. However, there was an interesting result in that there was some intense Mac3 staining of cells peripheral to the RANKL producing cell.

Inflammatory cytokine specific antibodies

The production of inflammatory cytokines in the interfacial membrane has been demonstrated and the IFM has been characterized as a zone of intense inflammation. The TNF- α antibody stained the RPGC well and in roughly the same pattern as the RANKL antibody. The TNF- α staining approximates the staining seen with the RANKL staining as seen in **figure 5A**. In contrast to the TNF- α staining, IL-1 β shows a much different staining pattern. This staining of RANKL in association with IL-1 β shows that IL-1 β is an intense signal in the middle of the RPGC approximately with several nuclei in the interior of the cell (**figure 5B**). The staining pattern of RANKL with IL-6 showed that while IL-6 is present in and around the RPGC, the IL-6 signal was low in intensity as compared to the other inflammatory cytokines. Though diminished, this IL-6 signal did appear on the outer edge of and within the RPGC indicating IL-6 was associated with the RPGC (**figure 5C**). All the inflammatory cytokines detected were bound with monoclonal antibodies and thus it would seem likely that all three inflammatory cytokines assayed are present in or on the RPGC indicating either the cell is bound by them or the cell is producing them in levels according to the intensity of the stain.

Cytomegalovirus antibody

The staining patterns seen on the RPGC indicate that it is of fibroblast origin and therefore raises the question as to how a fibroblast might form. The formation of a multi-nucleated fibroblast indicates cellular fusion of multiple mono-nucleated cells. There are several viruses of the herpes family that have been implicated in fibroblast fusion one of the most common of them being cytomegalovirus (CMV) [50]. We thus stained the

RPGC with a monoclonal antibody directed against a CMV protein. The CMV antibody did stain the RANKL producing cell though not in the same pattern as RANKL. The staining of CMV seems to associate with the Hoechst stain indicating that the epitope of the CMV antibody is in or in close proximity to the cell's nuclei as seen in **figure 6**. The staining was intense and approximated the shape of the cell as determined through DIC imaging. These data indicate that the RPGC is positive for CMV detection.

Figure 3: Presence of multiple RANKL positive giant cells in the interfacial membrane sample obtained from patient BJ020.

The figure shows several of these giant multi-nucleated RANKL positive cells in one field. Though these are present in all IFM samples so far observed they are usually fairly rare with this tissue being an exception. RANKL is denoted by the green color and the nuclei are denoted by the blue color. This image was taken at 400x magnification.

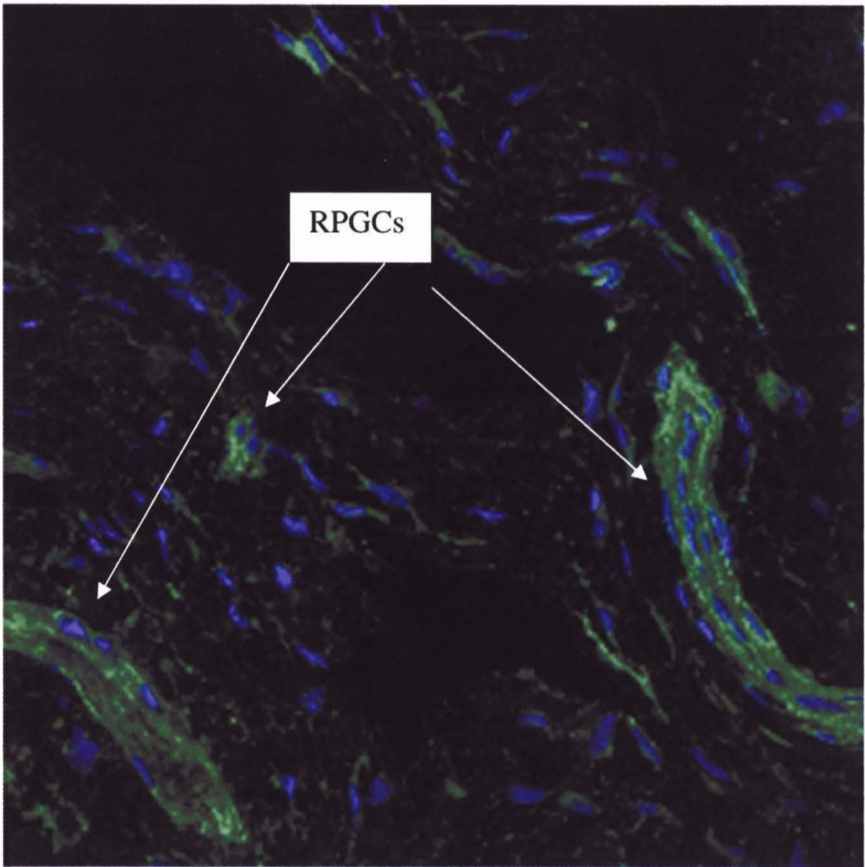


Figure 4: Observation of the RPGC using light microscopy. Panel a, shows the RANKL giant cell using the DIC filter and halogen lamp on the confocal microscope. Panel b, shows the same cell with the 405nm and 488nm laser activated to show fluorescence of RANKL (green) secondary and Hoechst nuclear stain (blue). Image taken at 630x.

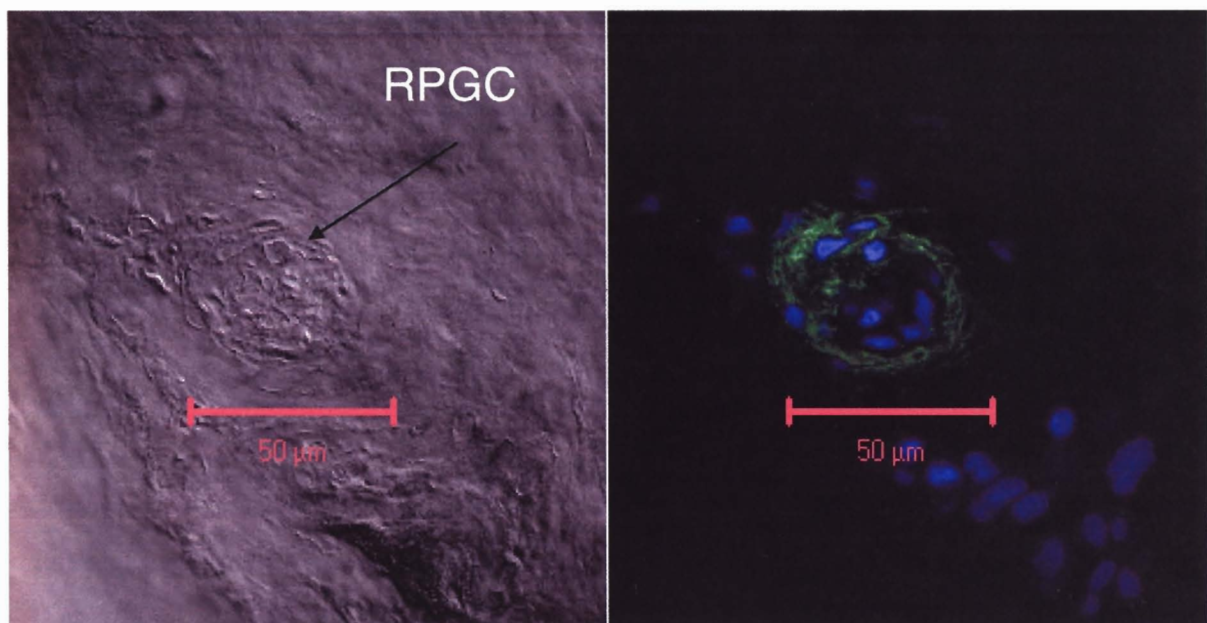
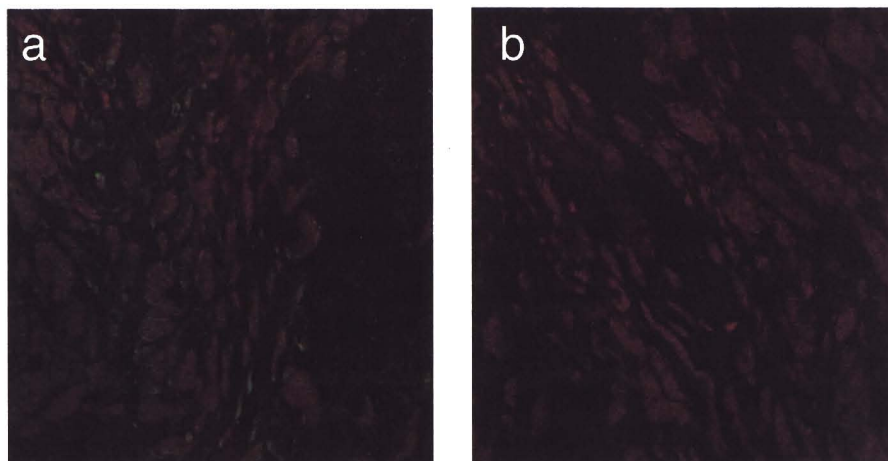


Figure 5: Controls of Case study.

Figure 5A. shows the secondary antibody controls for this experiment. Panel a, shows the anti-goat 488nm alexa fluor antibody. Panel b, shows the staining of anti-mouse 543nm alexa fluor antibody.

Figure 5B. shows the staining of the RANKL polyclonal and RANKL monoclonal antibodies. Panel a, shows the RANKL polyclonal antibody with anti-goat secondary (green). Panel b, shows the RANKL monoclonal antibody with anti-mouse secondary antibody (red). Panel c, shows a composite picture of both antibody stains combined with a Hoechst nuclear stain (blue). Image taken at 630x.

A.



B.

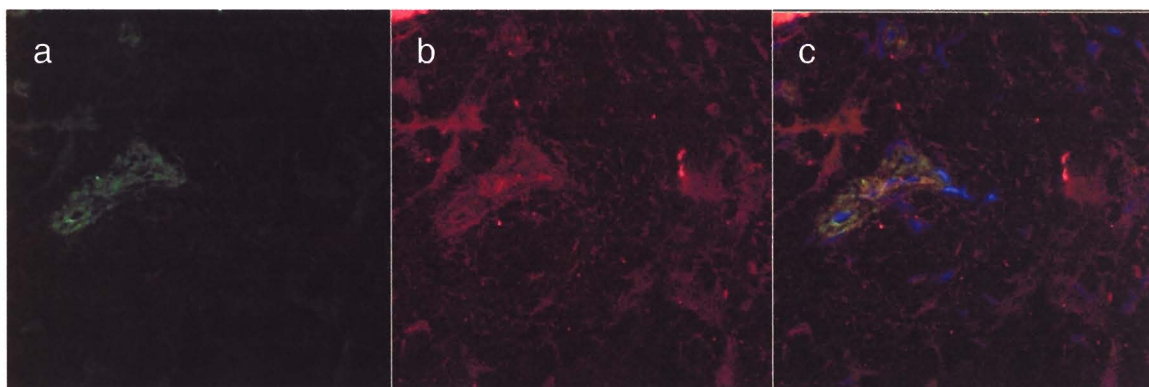
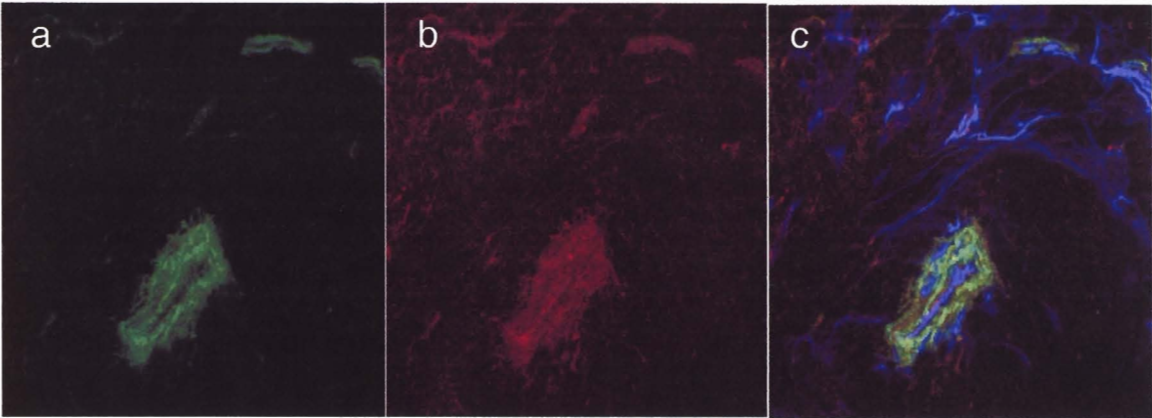


Figure 6: Staining of macrophage and fibroblast specific markers.

Figure 6A shows RANKL and D7-fib staining. Panel a, shows RANKL antibody staining with anti-goat secondary (green). Panel b, shows D7-fib antibody staining with anti-mouse secondary (red). Panel c, shows a composite picture of the two antibody stains with Hoechst nuclear stain (blue). Image taken at 630x.

Figure 6B shows RANKL and Mac3 staining. Panel a, shows RANKL antibody staining with anti-goat secondary (green). Panel b, shows Ber-Mac3 antibody staining with anti-mouse secondary (red). Panel c, shows a composite of the two antibody stains with Hoechst nuclear stain (blue). Image taken at 630x.

A.



B.

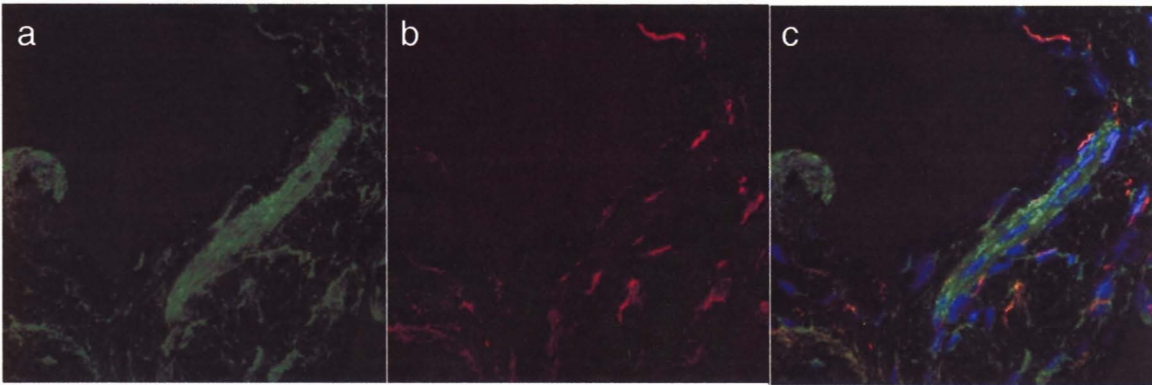


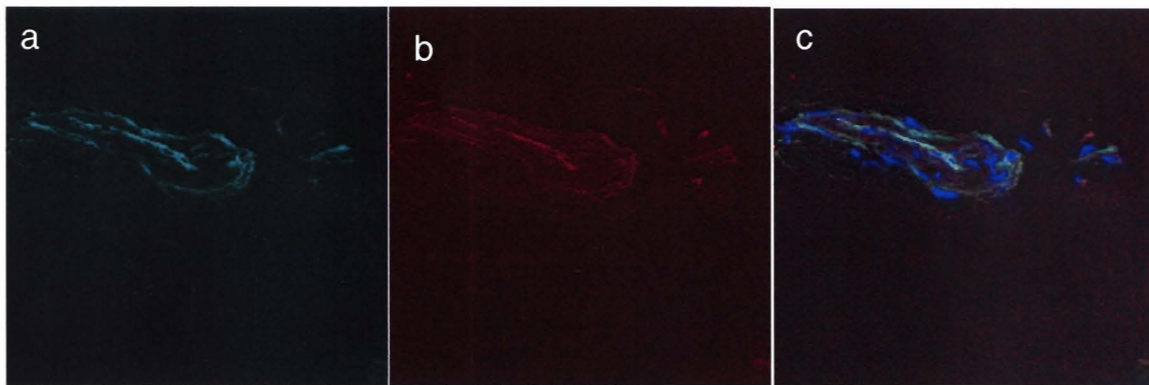
Figure 7: Staining of the RPGC with antibodies of inflammatory cytokines typically seen in the IFM.

Figure 7A shows the staining of the RANKL expressing cell with TNF-alpha antibody. Panel a, shows the RANKL antibody staining with anti-goat secondary (green). Panel b, shows the TNF-alpha antibody staining with anti-mouse secondary (red). Panel c, shows a composite picture of the two antibody stains with Hoechst nuclear stain (blue). Image taken at 630x.

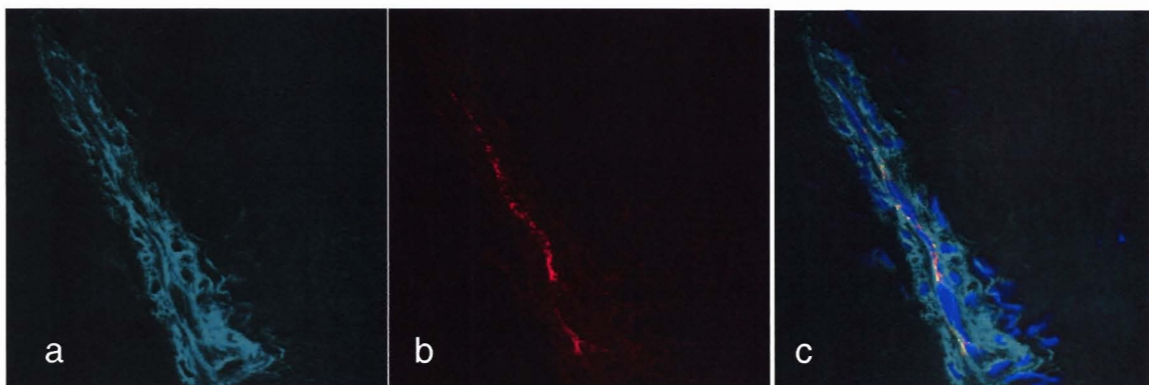
Figure 7B shows the staining of the RANKL expressing cell with IL-1. Panel a, shows RANKL antibody staining with anti-goat secondary (green). Panel b, shows IL-1 antibody staining with anti-mouse secondary (red). Panel c, shows a composite picture of the two antibody stains with together with Hoechst nuclear stain (blue). Image taken at 630x.

Figure 7C shows the staining of the RANKL expressing cell with IL-6. Panel a, shows RANKL antibody staining with anti-goat secondary (green). Panel b, shows IL-6 antibody staining with anti-mouse secondary (red). Panel c, shows a composite picture of the two antibody stains with together with Hoechst nuclear stain (blue). Image taken at 630x.

A.



B.



C.

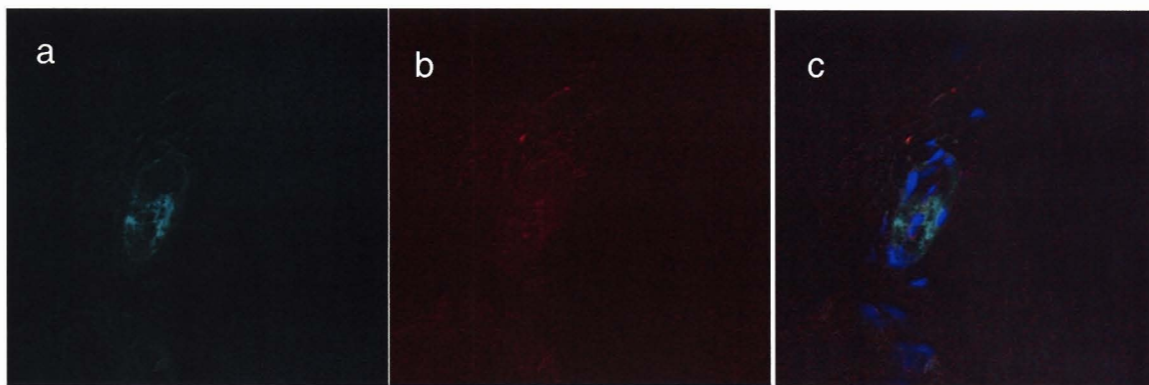
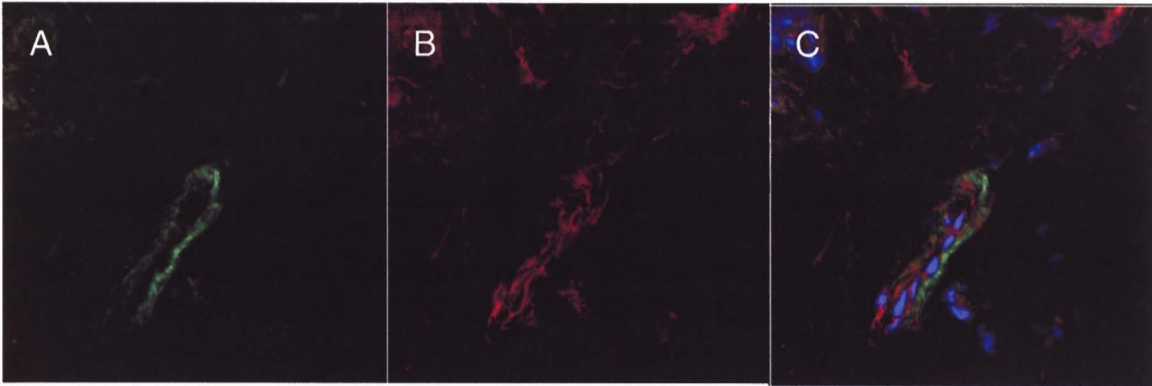


Figure 8: Staining of the RPGC with an antibody for cytomegalovirus.
Panel A, shows RANKL antibody staining with anti-goat secondary (green). Panel B, shows CMV monoclonal antibody staining with anti-mouse secondary (red). Panel C, shows a composite picture of the two antibody stains with together with Hoechst nuclear stain (blue). Image taken at 630x.



DISCUSSION

The formation of the interfacial membrane and its associated granuloma tissue is the sinequanon of the disease of aseptic osteolysis. We have examined this membrane to determine the cell source of the RANKL and in so doing we observed large multi-nuclear cells that express very concentrated levels of RANKL. RANKL is a potent membrane bound pro-bone reabsorbing cytokine. In this study we used various antibodies to cellular antigens and inflammatory cytokines in order to classify this RANKL producing cell. The study was performed on a 77 year old female's tissue that had an abundance of these cells present. The main antibody used for RANKL was a goat anti-human polyclonal specific to the extra-cellular domain of membrane intact RANKL.

The first antibody used was D7-fib. The purpose of this was to determine if the RANKL producing cell co-localized with D7-fib and thus was of fibroblast origin. The D7-Fib antibody recognizes a 112 kD molecule expressed on the cell surface of human fibroblasts and epithelial cells. The antibody does have the ability to bind to peripheral blood myeloid cells and in some cases smooth muscle. However in tissue it primarily targets fibroblast cells and epithelial cells. The antibody used in this study was a monoclonal antibody for D7 raised in mice and obtained from GeneTex. The staining of D7 with the RANKL closely approximated the same cellular pattern that the RANKL did. Because D7- fib antibody is specific to fibroblasts and the intense staining seen with D7-fib was so well co-localized with the RANKL antibody staining, these data indicate that the RANKL producing cell is fibroblast-like.

The formation of giant cells is not uncommon in the body and particularly with procedures involving prostheses implanted into the body. This has not only been

demonstrated with total joint arthroplasty [5, 55], but also with breast augmentation and the saline implants typically used in that procedure [56]. However foreign body giant cells (FBGC) are typically of myeloid origin thus it was necessary to stain the RANKL positive giant cell (RPGC) of this study with a myeloid markers in order to determine if this RPGC was of myeloid origin or not.

The antibody Ber-Mac3 is the human equivalent of murine Mac3 in that both are anti-CD163. CD163 is a member of the scavenger receptor super-family. In humans, CD163 mediates the uptake of hemoglobin-haptoglobin complexes. It is expressed by monocytes and macrophages. The Ber-Mac3 antibody was obtained from the DAKO Corporation and was raised as a monoclonal antibody in mice. The staining of the Ber-Mac3 antibody did not localize with the RANKL cell and showed no intensity on the cell denoted by the RANKL stain. However the Ber-Mac3 antibody did bind to cells surrounding the RANKL cell and showed some intensity there. This staining pattern is consistent with the current theory that pre-osteoclastic cells are myeloid in origin and must directly interact with RANKL producing cells in order to become mature osteoclasts. In previous co-localization from our laboratory the antibodies for RANKL receptor (RANK) and Ber-Mac3 have shown strong super-imposable co-localizing staining patterns (data not shown). This observation is consistent with the theory that Ber-Mac3 and RANK double positive cells surround a RANKL giant cell possibly interact with RANKL and receive pro-osteoclastic signals. This data is not by itself conclusive enough to confirm RANKL-mediated osteoclastogenesis, however, it provides sufficient evidence to suggest IFM macrophages are devoid of RANKL expression on their cell

surface, broadly supports our proposal that RPGCs of the IFM are not derived from the monocyte lineage.

Several reports by our group and by others document the expression of numerous inflammatory cytokines capable of regulating bone reabsorptive process in osteolysis namely, IL-1 β , IL-6 and TNF- α [12, 41, 42, 57]. The production of inflammatory cytokines by this RPGC was examined. TNF- α is one of the most common cytokines in the body and is produced by multiple cells in the body including fibroblasts and macrophages. Fibroblasts produce TNF- α in response to tissue damage and inflammation such as occurs in rheumatoid arthritis [58]. Macrophages, on the other hand, can respond to a plethora of foreign and natural factors by release of cytokine mediators. Particulate stemming from the metal, plastic or cement of prosthetic devices, as well as cell wall material of bacteria like LPS can activate IFM tissue macrophages to begin secreting cytokines. It is clear from a number of well controlled studies in the field of arthroplasty that macrophage activation and cytokine release is an important determinant of osteolysis [12, 33, 55]. TNF- α showed a staining pattern that was consistent with the general shape of the RPGC, as if it was present on its surface. These data suggests that either the RPGCs produce TNF- α or it has abundant soluble TNF- α bound to its surface.

IL-1 β is also a very common inflammatory cytokine and is very common to the interfacial membrane[12]. Interestingly, IL-1 β staining did not approximate the shape of the cell but was very intense in the cell interior. This observation could indicate this cell is actively translating IL-1 β in its cytoplasm but that it is secreting low levels of it or that the secreted IL-1 β was washed away. IL-1 β like TNF- α can be produced by fibroblasts in response to inflammation.

In contrast, RPGC stained the least intensely for IL-6. The staining pattern for IL-6 was very weak and did not localize well with the cell indicating this cell was producing low levels of IL-6 or that very little was bound to it. Prior work by our lab has shown the majority of IL-6 expression was in regions more highly populated by membrane fibroblasts than macrophages. This may indicate that the release of IL-6 from RPGC is highly efficient.

The inflammatory cytokine panel used in the present study indicates that the RPGC is capable of producing and it likely is actively producing inflammatory cytokines. Alternatively, the evidence might also suggest that the RPGC binds high levels of the cytokines. The staining pattern with IL-1 β seems to suggest that IL-1 β is being produced in the cell cytoplasm due to the intense staining inside the cell and no where else. The production of these cytokines is not inconsistent with this cell being of mesenchymal origin in that mesenchymal cells in an environment of inflammation are capable of producing IL-1 β and IL-6 [30, 59] Furthermore all three cytokines have been demonstrated as being produced by fibroblasts in response to particulate and bacteria [60].

The presence of a multi-nucleated fibroblast is certainly of some concern and raises the question as to what conditions would cause a multi-nucleated fibroblast to form. It has been documented that certain viruses of the herpes family can cause fusion of cells leading them to become multinuclear. One of these viruses in particular is cytomegalovirus (CMV). Garnett (1979) determined that CMV infection was capable of causing fibroblasts to fuse and form giant multinucleated cells [50]. We obtained a CMV monoclonal antibody from Chemicon International. This antibody was raised in mice

causing fibroblasts to fuse and form giant multinucleated cells [50]. We obtained a CMV monoclonal antibody from Chemicon International. This antibody was raised in mice with reactivity to human IE1-72 and IE2-86 proteins. IE1 and IE2 are immediate early proteins that are expressed in the first hour of infection and then over the entire course of CMV infection. Like most herpes viruses CMV goes through cycles of latency and active replication and like most other herpes viruses there is a high infection rate in the population with those infected experiencing repeated outbreaks from then on. The staining for CMV co-localized with the RANKL in each RPGC examined showing specificity and intensity of staining. These data document that the RPGCs in this patient are positive for CMV.

In order to control for false positive autofluorescence, we stained several sections with secondary antibody only. There was some staining noted and this can be attributed to the fact that this tissue is very carbohydrate dense and thus very sticky. As the methods demonstrate the tissues were washed stringently following incubations, however some non-specific binding was unavoidable. A final control used to validate our comparison data was use of a RANKL monoclonal antibody. Since RANKL was used in each staining it was necessary to confirm that the RANKL polyclonal antibody used was accurately binding RANKL. This RANKL monoclonal did in fact localize with the polyclonal indicating that both antibodies were adhering to RANKL and the polyclonal was providing accurate results. This result would validate the RANKL polyclonal binding pattern and indicate that there is significant RANKL being expressed on the membrane of this cell.

cellular structure and not a mass of fat or carbohydrate that bound antibody non-specifically. Using the DIC images as a guide, it was possible to confirm there was a cell-based structure present in the membrane that the antibody bound to specifically. The DIC images also give insight into the unique morphology of this cell; it does appear that this cell is not a normal portion of the tissue as the continuity of the tissue is disrupted by the cell structure. This supports the concept that the RPGC is not a normal structure, but rather a bizarre anomaly present in the IFM possessing high RANKL expression.

This case study represents the first of its kind to characterize a RPGC in the IFM of a periprosthetic osteolysis patient. Further studies will be done to further characterize the nature of this novel giant cell, and to determine if CMV plays a role in its formation. Presently it is sufficient to say that the RPGC produces a high concentration of RANKL and is a resident feature of the IFM. Furthermore, based on high powered confocal images, the RPGC appears to be multinucleated, is positive for fibroblast cell markers and is negative for markers of myeloid lineage. Based on this, we propose the RPGC is fibroblast-like cell type. This study identifies an important case history of osteolysis and an individual with multiple RPGCs present in the IFM. This study made use of greater resolution and detail of this cell type viewing this cell at 630x magnification as opposed to previous work by our laboratory that used 400x. Using transmission electron microscopy we will further try to increase the resolution and detail with which we see this cell and definitively answer the question of whether it is truly multi-nucleated or not.

CHAPTER 3

RESIDENT FIBROBLAST GIANT CELLS IN THE PERPROSTHETIC MEMBRANE ARE RANKL POSITIVE AND CO-LOCALIZE WITH CMV AND TACE

ABSTRACT

The previous work by this laboratory has demonstrated the presence in the IFM of a RANKL positive giant cell (RPGC) that also appeared multi-nucleated. This cell also stained positive for fibroblast markers. The purpose of this study was to better characterize this RPGC as a fibroblast through use of antibodies to fibroblasts and other cells typically found in the interfacial membrane.

We took interfacial membrane and associated granuloma tissue from five patients undergoing revision joint surgery due to osteolysis. This tissue was frozen sectioned onto slides and stained using fluorescent antibodies and observed using confocal microscopy. The presence of fluorescence was evaluated for intensity and localization with the RPGC.

The results of this study showed that the RPGC was negative for the osteoclast marker RANK and TRAP however, it was positive for the receptor to calcitonin. The RPGC stained positive for D7-fib, 5B5, and OPG while staining negative for osteocalcin and VDR. We were also able to demonstrate that this RPGC stains positive for cytomegalovirus and TACE.

The data suggests that the RPGC is of fibroblast origin while sharing similarities with osteoblasts and osteoclasts. It also appears that this cell is multi-nucleated and that its positive staining with CMV may offer an explanation as to the formation of a multi-nucleated fibroblast. Finally the positive expression of TACE co-localized with RANKL suggests that this RPGC is a source of RANKL to a widespread area of the IFM.

INTRODUCTION

Previous work done by our laboratory has identified a RANKL-positive multinucleated giant cell (RPGC) of fibroblast origin in the periprosthetic membrane of total joint arthroplasty patients. This cell has been observed in every interfacial membrane thus far studied, (approximately $n = 20$). RANKL is typically produced by osteoblasts, bone marrow stromal cells, and transiently by activated lymphocytes. The RANKL/RANK/OPG signaling system is a potent activator of bone reabsorption and high levels of RANKL detected in the interfacial membrane have shaped the idea that the interfacial membrane is not only a characteristic of periprosthetic osteolysis but also a mediator of it. The cell source of RANKL in the interfacial membrane is still unclear due to the absence of osteoblasts, however, recent data indicating IFM fibroblasts are capable of expressing RANKL and facilitating osteoclastogenesis in vitro has renewed interest in exploiting RANKL as a therapeutic target. Fibroblasts cultured out of the membrane and tissue have been shown to be positive for RANKL mRNA through RT-PCR [52]. The identification of a multinucleated RPGC expands the scope of RANKL-mediated osteolysis and thus this RPGC is of great interest. The purpose of this study was to better characterize the RPGC as being definitively a fibroblast or not. In addition to staining for fibroblast markers we stained for osteoblast markers and osteoclast markers. Sections from each patient's tissue were also stained with the light stain tartrate resistant acid phosphatase (TRAP) to demonstrate the presence of mature osteoclasts in each interfacial membrane sample. TRAP is an enzyme produced by mature osteoclast and osteoclast like cells and TRAP stain is a relied on method of detecting osteoclasts using light microscopy [19, 52].

This study involved five different interfacial membrane samples retrieved from five different patients undergoing revision joint surgery. The samples were collected over a period of time from June 2005 to March 2006. The membranes and associated tissue were collected from patients determined to have bone loosening around their prosthesis in the absence of infection as determined by clinicians. This panel of patients was not controlled for type of joint being revised and is a mix of hip and knee revisions. However, aseptic osteolysis has been demonstrated in both joints and formation of the IFM and granuloma is the common end result of the disease in each case [42]. The patients consisted of two males and three females and the ages ranged from 67 – 83 at the time of surgery. This study will attempt to determine whether or not the RPGC is of fibroblast origin or not using tissue from a varied cross-section of patients undergoing revision joint surgery due to aseptic osteolysis.

MATERIALS AND METHODS

Tissue preparation

Tissue was obtained from patients undergoing revision hip or knee surgery. The IFM and granuloma tissue was kept at 4° C following removal from the patient. The tissue was then embedded in OCT compound (Tissue-Tek) and frozen at -20°C until it was sectioned. The sections were made at 12 microns on a microtom frozen section cutter and melted on to superfrost microscope slides (Fisher Scientific). Frozen sections were kept at -20°C until stained.

Antibody Staining

Frozen sections were fixed using ice cold 100% methanol for 60 seconds and then washed with PBS. The slides were blocked and washed for ten minutes using a 3% goat serum and 0.1% triton PBS solution to decrease non-specific binding. Antibodies were diluted in PBS. Primary antibodies were diluted 1:50 and secondary antibodies were diluted 1:200 in PBS. Following each incubation the slides were washed 5 times with PBS to remove non-specific binding. All sections were stained with Hoechst 34580 (Molecular Probes) stain diluted 1:200 in PBS. **Table 3** summarizes the information about each antibody used in this study.

Analysis of Immunolabeling

Slides were observed using a Carl Zeiss LSM 510 Meta confocal imaging microscope. The lasers used for analysis were the 405nm diode laser, a 488nm argon laser, and a 543nm helium neon laser. All pictures were taken using a 63x oil immersion objective. In each case the characteristic intense RANKL staining was used to target cells for analysis. The sections were analyzed using FITC fluorescence to locate the

characteristic intense RANKL staining. Once located the examples were checked with light microscopy to ensure that a distinct cell was present. In all examples a Hoechst nuclear stain was used to determine if examples were multi-nucleated or not as this was a characteristic of previously examined cells.

TRAP staining

An IFM tissue section from each patient was stained for TRAP using a kit obtained from Sigma Aldrich. These sections were then counterstained with hemotoxylin. The sections were observed for TRAP⁺ cells using a 40x and a 100x objective on a standard light microscope connected to a digital camera. Upon observation of the sections giant cells were observed that appeared similar to Differential Interference Contrast (DIC) images taken of the RANKL producing cell during confocal microscopy. Images of these giant cells were captured in addition to TRAP positive cell images.

Table 3: Antibodies used in CMV and TACE study

The table summarizes the information on the antibodies used in this study including their name, host animal, fluorescent secondary and supplier.

Primary antibody	primary host animal	Secondary antibody	Supplier
CMV monoclonal	mouse	543 chicken anti-mouse	Chemicon International
RANKL polyclonal	goat	488 donkey anti-goat	Santa Cruz Biotechnology
RANK polyclonal	rabbit	543 chicken anti-rabbit	Santa Cruz Biotechnology
TACE monoclonal	rabbit	543 chicken anti-rabbit	Serotec Biological
D7-fib monoclonal	mouse	543 chicken anti-mouse	Gene Tex
OPG polyclonal	rabbit	543 chicken anti-rabbit	Santa Cruz Biotechnology
5b5 monoclonal	mouse	543 chicken anti-mouse	Dako Corporation
CTR monoclonal	mouse	543 chicken anti-mouse	Serotec Biological
Osteocalcin polyclonal	rabbit	543 chicken anti-rabbit	Santa Cruz Biotechnology
VDR polyclonal	rabbit	543 chicken anti-rabbit	Santa Cruz Biotechnology

RESULTS

Staining of RPGC with RANK and CTR antibodies

Besides foreign body giant cells (FBGC) in the IFM, another type of multi-nucleated cell that has been well characterized as being in the interfacial membrane is an osteoclast [5, 55]. Osteoclasts mediate bone reabsorption in the body through the breakdown of the bone matrix and the release of calcium. Osteoclasts express myeloid antigen surface integrins as they are of myeloid lineage, such as CD68, CTR and TRAP. The cell membrane bound receptor RANK is seen on pre-osteoclastic cells. Signaling through this receptor by RANKL causes osteoclastic differentiation and osteoclast activation. Additionally, the signaling of the RANK receptor provides an anti-apoptotic signal to mature osteoclasts. For this reason RANK is not only observed on pre-osteoclastic myeloid cells such as monocytes but also on mature osteoclasts. The sections were stained with two antibodies directed against osteoclastic antigen to determine if the RPGC was simply an osteoclast. The first antibody was against RANK. This antibody showed little to no staining in the section and did not stain the RPGC (**figure 9A**).

The second osteoclastic antigen we targeted was CTR. The CTR is expressed on osteoclasts and when bound by calcitonin it mediates a decrease in bone reabsorption. It is a good detector of osteoclasts in the IFM because while cells of the kidney and nervous system express CTR no other cell in and around the bone system expresses CTR. The staining of RANKL with CTR demonstrated significant binding of the CTR antibody with the RPGC. There was also significant staining of the surrounding tissue by the CTR antibody as can be seen in **figure 9B**.

Staining of RPGC with 5B5 and D7-fib antibodies

Previous studies have indicated that this giant RANKL producing cell stains positive for fibroblast markers [47]. In order to confirm this result and classify this RPGC as being fibroblast-like, we stained the tissue sections with the cell surface marker, D7-fib. In a case study done previously, we have seen that D7-fib co-localizes well with the RANKL antibody staining pattern and well approximates the shape of the RANKL producing cell. That result was observed again in this study. The D7-fib antibody stained well with the RANKL producing cell and localized well with the outline of the RANKL producing cell. The staining of D7-fib antibody was similar in pattern to that of the RANKL antibody. These two antibodies co-localized very well as seen in **figure 10A**.

In previous work on the multi-nucleated RANKL giant cell, we have seen D7-fib antibody positively binding to the RPGC and it was observed again in this study. In order to confirm this result and more conclusively determine that this cell was indeed a fibroblast we stained the sections with 5B5 antibody. 5B5 is an antibody that binds to the intracellular 4-prolyl-hydroxylase enzyme necessary for the production of collagen and thus is a good marker for identification of fibroblasts. 5B5 is used often in studies involving the IFM to identify fibroblasts [52]. The staining of the 5B5 antibody showed a splotchy pattern of staining with points of intense fluorescence and points of decreased fluorescence as compared to D7-fib. However, the staining of 5B5 antibody approximated the shape of the RANKL producing cell as denoted by RANKL staining. Despite a varied intensity along the RPGC for 5B5, the overall staining of 5B5 still gave intense fluorescence and was considered a positive result (**figure 10B**)

Staining of the RPGC with VDR, osteocalcin, and OPG

The expression of RANKL by osteoblasts is well documented and occurs under physiological conditions [8, 28]. The expression of RANKL by osteoblasts is up regulated by parathyroid hormone in response to low blood calcium level. The expression of RANKL by osteoblasts leads to an increase in osteoclast formation and activation. This increase in osteoclast activation will in turn increase bone reabsorption and release more calcium and phosphate into the blood. The fact that osteoblasts express RANKL made it necessary to determine if the RPGC was positive for antibody staining specific to osteoblasts.

The first osteoblast-specific antibody used was against the vitamin D receptor (VDR). Vitamin D is responsible for increasing calcium levels in the blood through action on the GI tract, kidneys, and bone. The presence of VDR is most highly seen in the villi of the duodenum, distal tubule cells of the kidney, and cells of the bone specifically osteoblasts and osteocytes. The active form of vitamin D, calcitriol, is involved in mediating osteoblastic expression of RANKL which will increase osteoclastogenesis and increase blood calcium levels. While calcitriol is seen in most all cell types in the body it is expressed only in low levels in areas other than the intestine, kidneys, and skeletal system. The staining of VDR with the RANKL giant cell revealed weak staining of VDR antibody on the RPGCs (**figure 11A**). This low level of staining indicates that VDR is undetectable or very low in the RPGC.

The non-collagenous protein osteocalcin is produced by osteoblasts in response to calcitriol. It is a protein unique to bone tissue that causes the proper deposition of phosphate and calcium in the osteoid during new bone formation. The staining of the

IFM tissue samples with osteocalcin revealed rather intense staining over the entire tissue. However, the important observation was that the RPGC demonstrated only limited staining with osteocalcin antibody as compared to the rest of the tissue. In fact the cell was relatively absent of osteocalcin staining and certainly showed a decreased intensity when compared to the surrounding tissue (**figure 11B**). These data would suggest that the RPGC is negative for osteocalcin.

The third part of the RANKL/RANK signaling triad is the molecule osteoprotegrin (OPG). OPG is expressed in similar cell types as RANKL, such as osteoblasts, however, OPG gene expression can be reciprocal to that of RANKL. The function of OPG is the inhibition of RANKL signaling through blocking of the interaction between RANKL and RANK. OPG is a soluble decoy receptor of RANKL typically expressed as regulatory measure by RANKL producing cells. The antibody staining for OPG showed intense co-localization with the RPGC. The antibody for OPG approximated the same staining pattern as the RANKL antibody and co-localized with the RANKL antibody as seen in **figure 11C**. It seems likely that this cell is secreting both OPG and RANKL.

Staining of the RPGC with TACE and CMV antibodies

The presence of a multi-nuclear fibroblast is an interesting phenomenon that is certainly not normal under physiological conditions in the body. In order to give some explanation as to how a multi-nuclear fibroblast might form we investigated the possibility of viral mediated cellular fusion. Based on information from a virologist associated with our laboratory we determined that human cytomegalovirus (CMV) might be a likely candidate as a mediator of fibroblast cell fusion. CMV has been demonstrated

in vitro and in vivo to cause the fusion of fibroblasts into multinuclear giant cells [50]. Clinical observations of CMV infected tissues have revealed swollen multi-nuclear cells that could be similar to the giant cell we observe in the IFM. The tissue sections were thus stained with a CMV monoclonal antibody to determine at least if these cells were CMV antibody positive. The staining pattern showed co-localization of the CMV antibody with the RPGC in all examples looked at. The signal from the CMV antibody was very strong in most of the examples and certainly present in all of them (**see figure 12 A and B**). The staining was not always consistent with the RANKL outline of the RPGC as it sometimes was more localized to the nuclei of the cell however the CMV antibody was definitively attached to the cell in each case.

The enzyme TNF-alpha converting enzyme (TACE) has been implicated in the formation of soluble RANKL. The enzyme is membrane bound and clips off the RANKL molecules from the cell expressing it and releases them into the extra-cellular environment. Recent studies have shown that this soluble RANKL is in fact active and capable of inducing osteoclastogenesis [61-63]. The presence of TACE co-localized with the RPGC would indicate that this cell that so highly expresses RANKL is capable of mediating pro-osteoclastogenic effects to a much greater population of cells than those immediately surrounding it. When the tissue sections were stained with TACE it was observed that there was intense staining and co-localization of the TACE antibody with RPGC. When compared to the other assays done in this study TACE represented the highest intensity of staining and thus antibody binding of all the antibodies used other than RANKL (**figure 12 C**). These data depict the RPGC as TACE positive and as a co-localized signal with RANKL.

TRAP staining of IFM

In all IFM tissue samples examined, TRAP positive cells were observed. TRAP is an enzyme produced by osteoclasts as they mature and after they become activated. The significance of the TRAP stain indicates the osteoclasts are present in the membrane. However, due to the method of TRAP staining which requires counter staining with hematoxylin we observed that multi-nucleated TRAP negative cells could be observed using the light microscope. The observation that RPGCs stained negative for TRAP is consistent with the concept that RPGCs are non myeloid entities (**figure 13**)

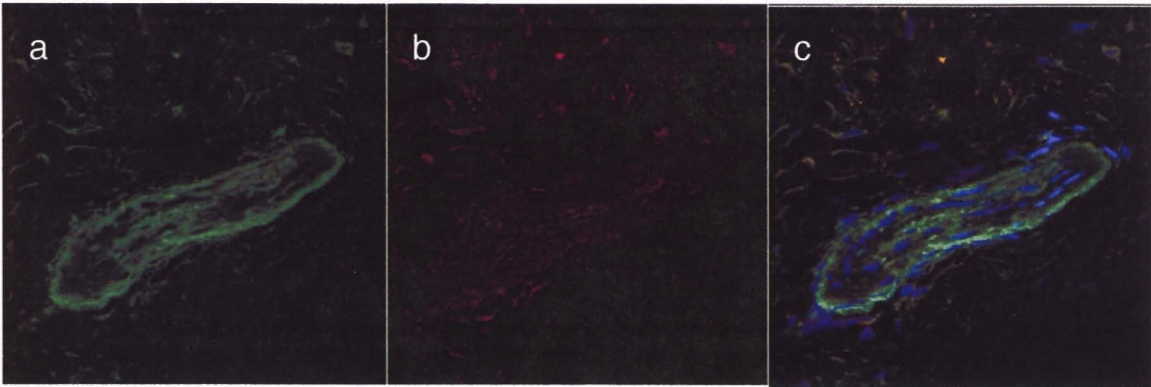
Figure 9: The staining of the RPGC with RANK and CTR antibodies

Figure 9A: Panel a, shows the RANKL antibody staining with anti-goat secondary (green). Panel b, shows RANK antibody staining with anti-rabbit secondary (red). Panel c, shows a composite picture of the two antibody stains with Hoechst nuclear stain (blue).

Image taken at 630x. (BJ020)

Figure 9B: Panel a, shows the RANKL antibody staining with anti-goat secondary (green). Panel b, shows the CTR antibody staining with anti-mouse secondary (red). Panel c, shows a composite picture of the two antibody stains with Hoechst nuclear stain (blue). Image taken at 630x. (BJ022)

A.



B.

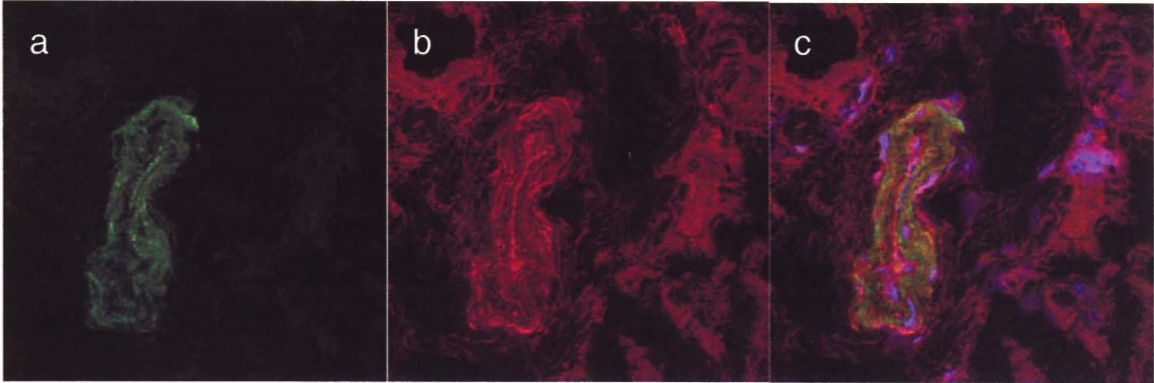
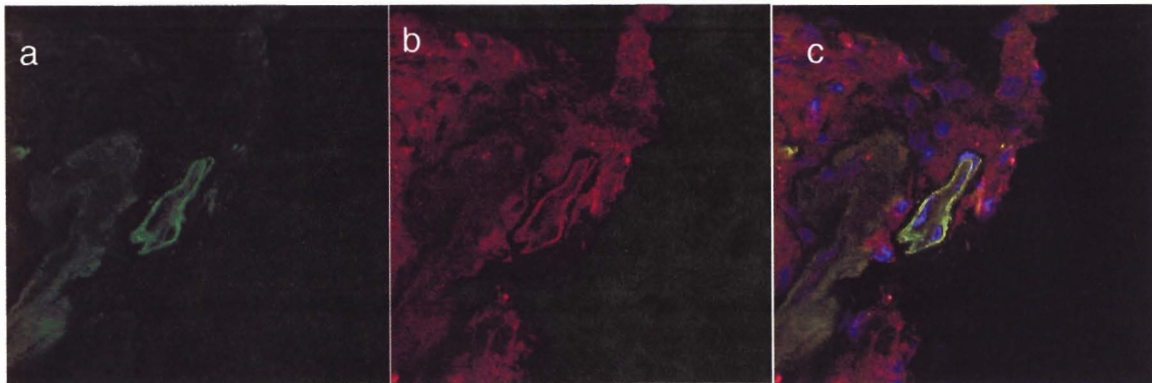


Figure 10: Staining of the RPGC with D7-fib and 5b5 antibodies

Figure 10A: Panel a, shows the RANKL antibody staining with anti-goat secondary (green). Panel b, shows the D7 antibody staining with anti-mouse secondary (red). Panel c, shows a composite picture of the two antibody stains with Hoechst nuclear stain (blue).
Image taken at 630x. (BJ018)

Figure 10B: Panel a, shows the RANKL antibody staining with anti-goat secondary (green). Panel b, shows the 5B5 antibody staining with anti-mouse secondary (red). Panel c, shows a composite picture of the two antibody stains with Hoechst nuclear stain (blue).
Image taken at 630x. (BJ020)

A.



B.

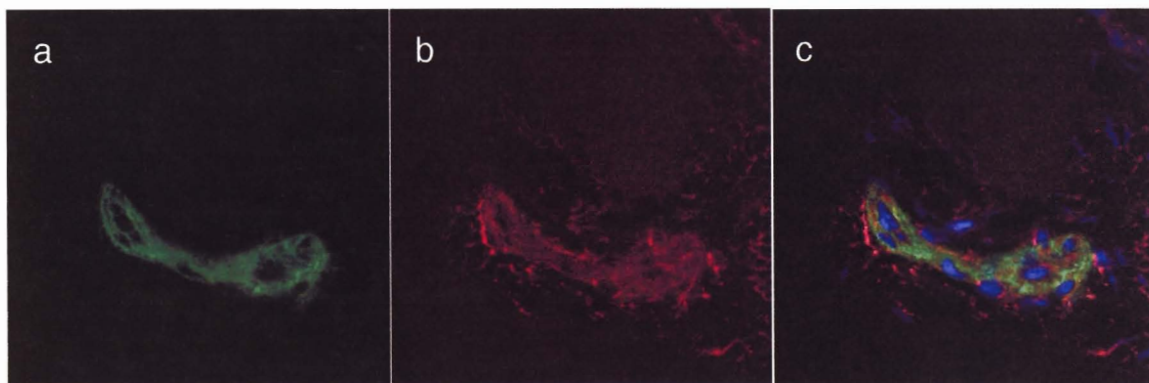


Figure 11: Staining of the RPGC with VDR, osteocalcin, and OPG antibodies

Figure 11A: Panel a, shows the RANKL antibody staining with anti-goat secondary (green). Panel b, shows the VDR staining with anti-mouse secondary (red). Panel c, shows a composite picture of the two antibody stains with Hoechst nuclear stain (blue).

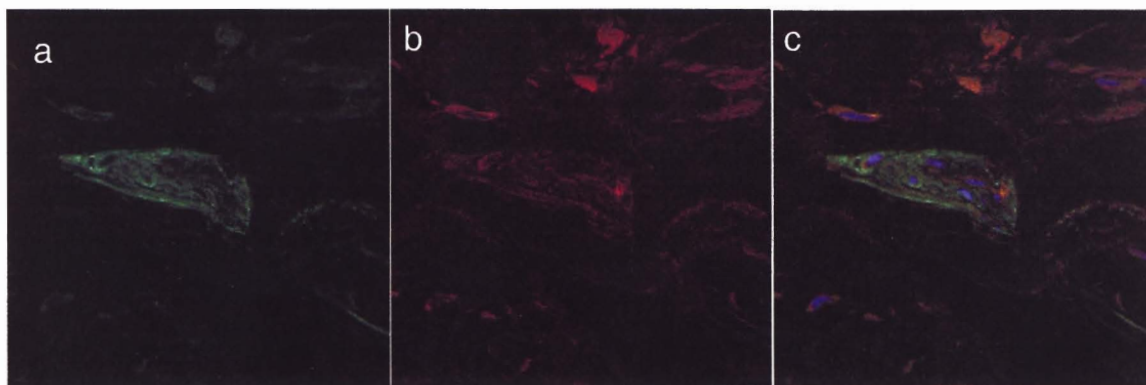
Image taken at 630x. (BJ005)

Figure 11B: Panel a, shows the RANKL antibody staining with anti-goat secondary (green). Panel b, shows the osteocalcin antibody staining with anti-mouse secondary (red). Panel c, shows a composite picture of the two antibody stains with Hoechst nuclear stain (blue). Image taken at 630x. (BJ005)

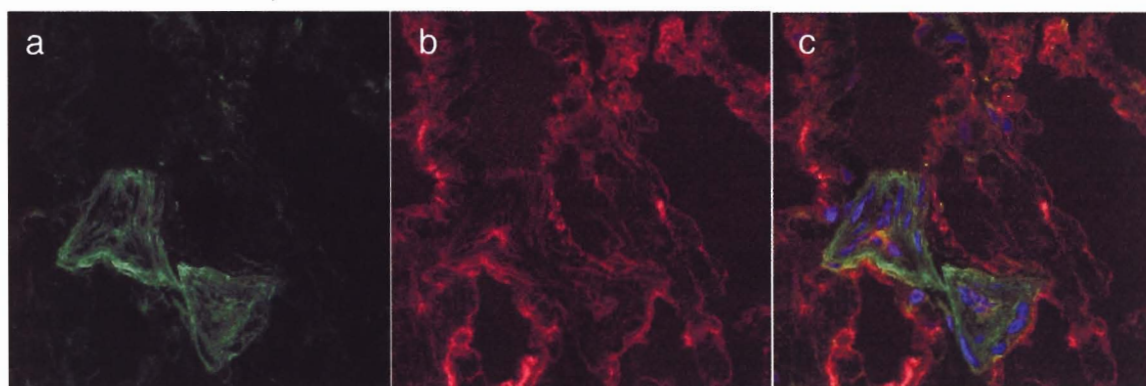
Figure 11C: Panel a, shows the RANKL antibody staining with anti-goat secondary (green). Panel b, shows OPG antibody staining with anti-rabbit secondary (red). Panel c, shows a composite picture of the two antibody stains with Hoechst nuclear stain (blue).

Image taken at 630x. (BJ018)

A.



B.



C.

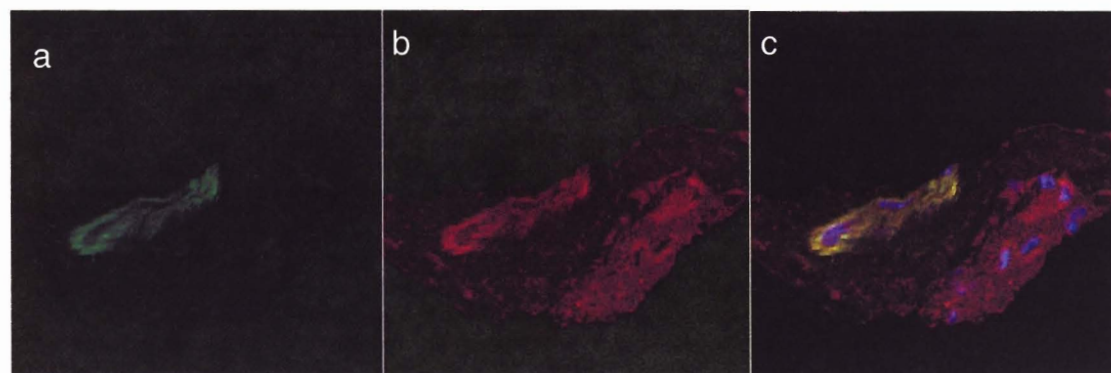


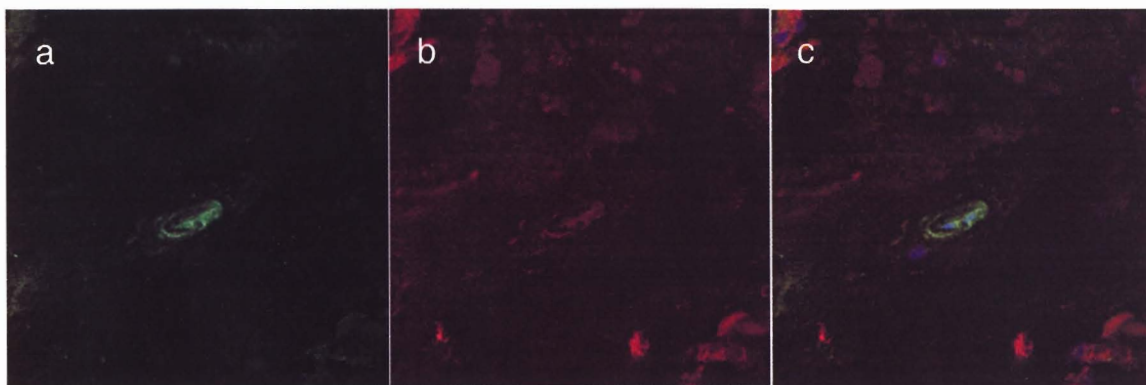
Figure 12: Staining of the RPGC with CMV and TACE antibodies.

Figure 12A: Panel a, shows the RANKL antibody staining with anti-goat secondary (green). Panel b, shows the CMV antibody staining with anti-mouse secondary (red). Panel c, shows a composite picture of the two antibody stains with Hoechst nuclear stain (blue). Image taken at 630x. (BJ021)

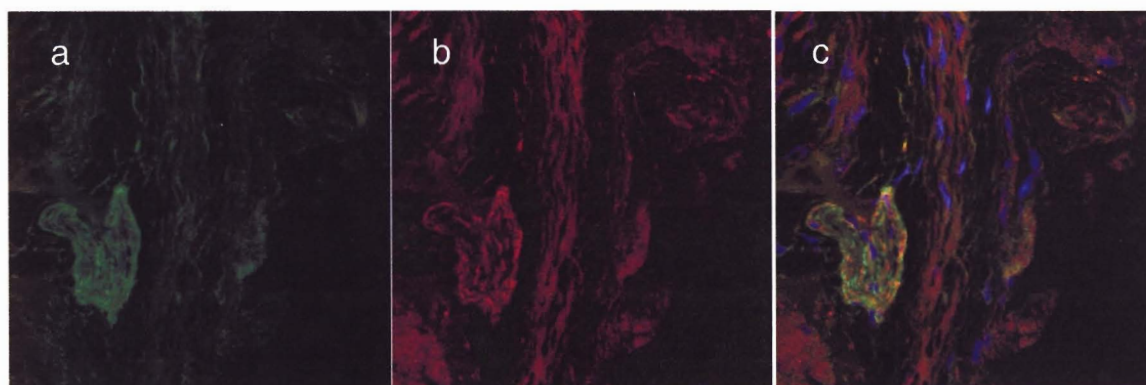
Figure 12B: Panel a, shows the RANKL antibody staining with anti-goat secondary (green). Panel b, shows the CMV antibody staining with anti-mouse secondary (red). Panel c, shows a composite picture of the two antibody stains with Hoechst nuclear stain (blue). Image taken at 630x. (BJ018)

Figure 12C: Panel a, shows the RANKL antibody staining with anti-goat secondary (green). Panel b, shows the TACE antibody staining with anti-mouse secondary (red). Panel c, shows a composite picture of the two antibody stains with Hoechst nuclear stain (blue). Image taken at 630x. (BJ020)

A.



B.



C.

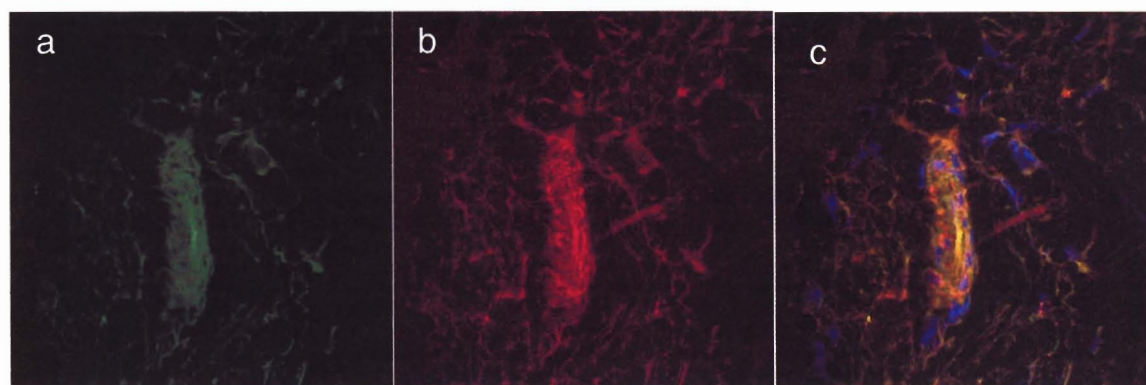
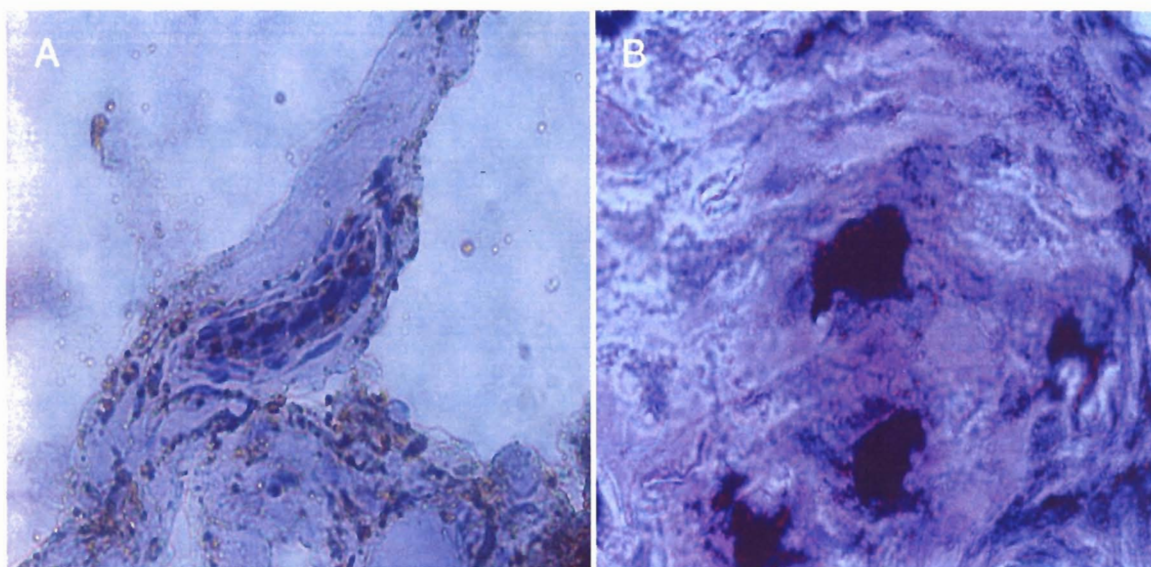


Figure 13: TRAP staining of IFM

Panel A, shows the multinucleated cell stained with hematoxylin at 400x (BJ020)

Panel B, shows the presence of TRAP⁺ cells in the IFM at 1000x (BJ022)



DISCUSSION

The formation of the interfacial membrane and granuloma is a common end product of all aseptic osteolysis. RANKL is a potent activator of bone reabsorption. We have studied this membrane to identify the cell source of the RANKL and through doing this we observed large multi-nuclear cells that express very high levels of RANKL. RANKL is typically produced by osteoblasts however in the interfacial membrane RANKL has been demonstrated to be expressed by fibroblasts [9, 52]. The purpose of this study was to distinguish this RPGC seen in all IFM samples as being definitively of fibroblast origin. Additional assays were done to determine a possible hypothesis explaining the formation of this cell and how it might be important in the aseptic osteolysis story. The study was done using tissue obtained from five different patients undergoing revision joint surgery. The tissue was subjected to antibody staining and then confocal microscopy. Several tissue sections were stained with TRAP and evaluated using light microscopy.

The first step in defining this RPGC was to eliminate possibilities of other cells it could be. Multinuclear giant cells are seen in the interfacial membrane in the form of osteoclasts. Osteoclasts do not express RANKL however they do express RANK and if there were soluble RANKL present that was bound to this cell it could be possible for an osteoclast to stain positive for RANKL [14]. Thus it was necessary to stain this RPGC with osteoclast markers to determine whether or not it was an osteoclast.

The first antibody was directed against the membrane bound receptor RANK. RANK is a member of the TNF receptor superfamily and when bound it interacts with the protein TNF Receptor Associated factor (TRAF) to activate NF- κ B. RANK has been

shown recently to be expressed on pre-osteoclastic cells and its activation leads to the formation and activation of osteoclasts [14]. RANK is expressed on most cells of myeloid lineage. The antibody for RANK was a polyclonal antibody raised in rabbits against amino acids 317-616 mapping to the C-terminus of human RANK. The staining with RANK showed no co-localization with the RANKL producing cell and no intensity. The absence of RANK on the RANKL cell gives evidence to the fact that it is not of myeloid lineage and also is not an osteoclast.

To further evaluate the possibility that the RPGC was an osteoclast, tissue sections were also stained with an antibody to CTR. The CTR molecule is expressed in kidney cells, central and peripheral nervous system cells and osteoclasts. The CTR is a cell surface membrane protein with seven transmembrane regions coupled to a G protein. The binding of calcitonin to its CTR on osteoclasts causes a decrease in their activity and a corresponding decrease in bone reabsorption. The molecule calcitonin is a 32 amino acid peptide generated from parafollicular cells in the thyroid gland. The purpose of calcitonin is to regulate calcium and phosphorus levels in the blood. It was a monoclonal antibody raised in mice against a cytoplasmic epitope of the seven transmembrane region of the receptor. The positive binding of CTR antibody to the RPGC does seem to weaken the argument for this cell not being an osteoclast. However, the failure of the cell to bind with RANK and failure of this cell to bind Ber-Mac3 in previous experiments (data not shown) would suggest that this cell is not of myeloid lineage as an osteoclast would be. Furthermore, there has been evidence recently that foreign body giant cells express CTR though are not osteoclasts [55, 64, 65]. It is possible that this RPGC shares characteristics with other giant cells found in the interfacial membrane though its failure to bind myeloid

markers make it unlikely to be a foreign body giant cell. The expression of RANKL, therefore, is not a characteristic of osteoclasts or foreign body giant cells [65].

The antibodies designed to denote the cell as fibroblast were 5B5 and D7-fib. 5B5 is an antibody designed to bind to the prolyl 4-hydroxylase protein that is seen in fibroblasts actively synthesizing collagen. Prolyl 4-hydroxylase is an enzyme that is central to collagen production. It catalyzes the formation of 4-hydroxyproline by hydroxylation of prolines in X-pro-Gly sequences on collagens, this process of course being essential to the formation of collagen triple helix. The antibody used in this study was monoclonal for prolyl 4-hydroxylase and raised in mice against human antigen. This antibody co-localized well with the RPGC in spotty though distinct pattern that approximated the shape of the entire cell. The cell that both RANKL and 5B5 stained contained multiple Hoechst stain positive spots which would indicate nuclei. The staining pattern seen in this cell would indicate that the RPGC is positive for prolyl 4-hydroxylase which would certainly point to it being a fibroblast.

The second fibroblast marker used was D7-fib. D7-Fib is an antibody that recognizes a 112 kD molecule expressed on the cell surface of human fibroblasts and epithelial cells. The antibody does have the ability to bind to peripheral blood myeloid cells and in some cases smooth muscle. However, in tissue it primarily targets fibroblast cells and epithelial cells. The antibody used in this study was a monoclonal antibody for D7-fib raised in mice. The staining of D7-fib with the RPGC was very good at approximating the shape of the cell and had a similar staining pattern to the RANKL antibody however in some examples it did lack intensity in its staining. Despite this observation, the similar staining of the RANKL antibody and the D7-fib antibody would

indicate this cell is positive for the D7-fib antigen and is of fibroblast origin. When taken in concert with the observations of the 5B5 staining, it seems very likely that this cell is a fibroblast. As with the previous stains the RANKL staining cell had multiple nuclei in it. This is characteristic of these cells and was used in addition to RANKL expression to target these cells for analysis.

The expression of RANKL is not a uniquely an osteoblast characteristic, however, in the bone system they are the dominant producer of it. Osteoblasts produce high levels of RANKL in response to parathyroid hormone and calcitriol. Studies of the composition of the interfacial membrane have revealed that very few osteoblasts are present there [12, 42]. However, this RPGC is also relatively rare in the tissue which would make it conceivable that it was an osteoblast-like multi-nucleated RANKL expressing cell. In order to test this hypothesis the tissue sections were stained with three different antibodies to antigen expressed by osteoblasts.

VDR is typically expressed on distal tubule kidney cells, villi of the duodenum, osteocytes and osteoblasts in high concentration with low concentrations seen in most other cell types of the body. The purpose of the VDR is to mediate the effects of calcitriol. The primary function of calcitriol is to maintain homeostatic levels of calcium in the blood. Calcitriol functions on the cells of the duodenum to increase calcium absorption from the digestive track. Similarly when calcitriol acts on the osteoblasts it increases their production of RANKL for the purposes of increasing bone reabsorption which increases calcium and phosphate levels in the blood. The VDR antibody used was a rabbit polyclonal raised against the C-terminus of VDR in rats. This antibody shows significant cross-reactivity with mice and humans. Calcitriol can up or down regulate

cells responses including proliferation, differentiation, and calcium homeostasis by acting at the level of gene transcription. The limited staining with VDR antibody indicated the RPGC is distinct from an osteoblast, which would contain a strong signal for VDR. These data suggest that this RPGC expresses low levels of VDR, if any at all.

Osteocalcin is a 5800 MW extrahepatic vitamin K dependent protein uniquely expressed by osteoblasts. Osteocalcin is one of the most abundant proteins in the body and the second most abundant protein in the skeletal system next to collagen. Osteocalcin functions in facilitating the binding of calcium ions and hydroxyapatite together to be deposited on the collagen matrix of developing bones. Osteocalcin is a highly conserved 46-50 amino acid single chain protein that contains three vitamin K-dependant gamma-carboxyglutamic acid residues. The antibody was a polyclonal antibody raised in rabbits against the full chain of human osteocalcin. The staining pattern with osteocalcin showed intense staining of the surrounding interfacial membrane. The stain however showed only small portions of co-localization with the RANKL producing giant cell. The staining was in fact diminished on the giant cell with regards to the surrounding tissue. The intense staining of the surrounding tissue can possibly be attributed to either high level of osteocalcin in the cells of the IFM or a potentially high levels of bone reabsorption as would be noted in osteolysis. The staining seen in this example could be representative of fragments of osteocalcin being trapped in the surrounding tissue of an area where high levels of bone reabsorption are taking place; the IFM is such a tissue. Additionally, the osteocalcin antibody was raised against the entire osteocalcin molecule which would make it possible to bind to fragments of the original protein that have become trapped in the tissue. The RPGC did have some binding with the osteocalcin antibody but it was

reduced compared to that of the surrounding tissue. This indicates that this RPGC is negative for osteocalcin production.

The final antibody used that was specific to an osteoblast antigen was against OPG. OPG is the abbreviation of the protein osteoprotegrin and is also known as osteoclastic inhibition factor (OCIF). OPG is expressed by RANKL producing cells such as osteoblasts as a form of self regulator of RANKL signaling. The OPG antibody used in this study was an anti-human polyclonal raised in rabbits against amino acids 153-401 of OPG. The staining with OPG showed some intense staining on the RPGC and co-localized well with the cell. In some examples this stain appeared to be more intense around the nuclei of the cell than the RANKL stain, however, it was consistently seen on the RPGC. These data provide evidence that the RPGC could be of osteoblast origin. However, it has been well documented that fibroblasts isolated from the interfacial membrane in addition to expressing RANKL are capable of expressing OPG [55]. OPG production and RANKL production are consistently detected in fibroblasts removed from the interfacial membrane [52]. To this effect, the staining pattern of the OPG stain does not strengthen or weaken the argument that this multi-nucleated cell is fibroblast-like. It simply strengthens the argument that this cell shows similar properties to other RANKL producing cells.

In addition to the fluoro-stains to characterize the RPGC as either osteoblast or fibroblast, we stained IFM tissue sections from all patients with a CMV antibody. This was a result of a previous case study done in this laboratory where CMV antibody bound successfully to the RPGC. The positive CMV antibody staining in one patient facilitated the need to repeat the experiment in multiple patients in an effort to demonstrate the

presence of CMV in this cell. It has been documented that certain viruses of the herpes family can cause fusion of cells leading them to become multinuclear. One of these viruses in particular is cytomegalovirus (CMV) [49]. As stated previously, CMV infection has the capability of causing fibroblasts to fuse and form giant multinucleated cells [50]. We obtained a CMV monoclonal antibody raised in mice with reactivity to human IE1-72, and IE2-86 CMV epitopes. IE1 and IE2 are early non-structural antigens of CMV infected cells that can be detected after 1 hour of infection, but can be detected throughout the entire CMV infection cycle. Like most herpes virus family members, CMV goes through cycles of latency and active replication and like most other herpes viruses there is a high infection rate in the population and the immune system can never quite clear the disease [49]. The staining for CMV very well co-localized the RANKL cell and demonstrated some intensity of staining. These data show that the RANKL cell is CMV antibody positive. Although more testing will need to be done to determine CMV's involvement in the formation of this cell, the positive antibody staining certainly provides a plausible hypothesis that CMV infection of these fibroblast-like cells in the IFM causes them to fuse forming multi-nuclear cells that produce high levels of RANKL.

RANKL is a membrane bound molecule that interacts with RANK to mediate osteoclastogenesis. There has been evidence recently that soluble RANKL molecules may be a viable source of the RANKL signal and thus bypass the essential need for the cell to cell interaction that is modeled in bone tissue. Soluble RANKL could allow a RANKL producing cell to mediate its effects over a much broader area of tissue than those cells immediately adjacent to it. The formation of soluble RANKL has been associated with the enzyme TACE, which stands for TNF-alpha converting enzyme.

TACE, also known as cd156b, is a membrane-bound metalloprotease disintegrin that belongs to the mammalian ADAM family. TACE has the ability to clip the RANKL molecule from the cellular membrane to which it is bound and allow it to be secreted into the extracellular environment [66, 67]. This secreted RANKL has recently been characterized as being capable of inducing osteoclastogenesis and stimulating bone reabsorption [61, 62]. The TACE antibody was raised in rabbits against amino acids 807-823 at the C-terminal end of human TACE. The antibody staining of TACE was the most intense staining seen in this study next to RANKL. It co-localized exceptionally well with the RANKL antibody and gave a very intense signal. These data strongly suggest that these giant RANKL producing cells are also producing TACE. The co-production of TACE and RANKL would lead to a scenario where you have an abundant production of soluble RANKL which would allow this cell to disperse pro-bone reabsorbing signals over a wide area in the membrane.

The staining of the IFM granuloma tissue with TRAP demonstrated the identification of multiple TRAP⁺ cells in the IFM tissue samples. TRAP is produced by osteoclasts both prior and after they reach maturity. The presence of TRAP⁺ cells indicates the presences of osteoclasts in the interfacial membrane. Additionally, as a by-product of the TRAP staining which required hematoxylin counter-staining we were able to observe the giant cells under a light microscope without the aid of immunofluorescence. The RPGCs were not positive for TRAP which when coupled with the antibody stain against RANK, only serve to strengthen the argument that these multi-nucleated cells are not osteoclasts but rather some other type of cell. Based on the antibody staining and composition of the membrane it would seem likely that this cell is a fibroblast.

Additionally, the visualization of this cell using a different technique than confocal microscopy gives validity to the cell's presence in the IFM and insight into its morphology.

More detailed imaging, such as electron microscopy, will be required to determine if it is really a multi-nucleated cell or simply an aggregate of cells close together. However, the fact remains that this structure produces a high level of RANKL and its antibody staining is consistent with it being a fibroblast. Electron microscopy is needed to determine whether the cell is multinucleated or not and at present a sample is being prepared for transmission electron microscopy. The RANKL producing giant cell seen in the interfacial membrane stained positive for CMV antibody. The infection of this cell with CMV could lead to an explanation of how it fuses. This cell appears to be a strong source of RANKL in the interfacial membrane and as such could be a potent activator of osteoclastogenesis and cause osteolytic bone reabsorption. This is supported by the evidence of high levels of TACE on the giant cell. Should this giant cell be producing high levels of RANKL and high levels of TACE then it is possible for this cell to mediate pro-osteoclastogenic events in a wide area of tissue. The high level of RANKL and TACE being expressed on this cell would indicate that while rare in the tissue this cell can be an important activator of osteoclastogenesis and osteoclast activation leading to osteolysis. Future research efforts in regards to this cell will involve attempts to isolate this RANKL producing cell from the interfacial membrane using laser capture microscopy with the intention of performing RT-PCR on it. Analysis of the RNA present in the cell through RT-PCR will give insight into the genes in the cell actively being transcribed. Analysis of these transcripts can give insight into the role TACE plays

in the RPGC's effectiveness as a mediator of osteolysis. The RNA analysis will also be able to determine what role CMV plays in the formation of this cell. Should it be determined that this RPGC is a potent mediator of aseptic osteolysis and it is formed by CMV then it could lead to new clinical options in the prevention of osteolysis. Currently, preventive anti-viral drugs are given to graft recipients to combat CMV and potentially this could become an anti-osteolysis therapy [68]. Although due to the toxic nature of these drugs this seems like an unlikely option. The most promise for osteolysis therapy would seem to lie with anti-RANKL treatments as these are already in clinical trials.

GENERAL CONCLUSIONS

The data from the two studies presented in this thesis demonstrate that there is a large structure in the IFM that is RANKL positive and multi-nucleated. The RPGC was not of myeloid origin as demonstrated by failure to bind RANK or BerMac3. The RPGC was positively identified using fibroblast-specific antibodies, D7-fib and 5B5. Thus, these data support the hypothesis that the RPGC is a multi-nucleated cell and fibroblast in origin. However, the binding of the RPGC with VDR, RANKL, and osteoclastin suggest that this RPGC shares some characteristics with osteoblasts. The negative TRAP staining indicates that RPGCs are not an osteoclast however the binding of CTR demonstrates this RPGC does have a similarity with osteoclasts and foreign body giant cells. The positive binding of inflammatory cytokines to this cell provide evidence that RPGCs mediate inflammation in the IFM through cytokine production or responds to the inflammation through the high concentrations of cytokines bound to it. This RPGC also showed a positive identification for CMV and TACE. We hypothesize that this RPGC is a multi-nucleated fibroblast formed through infection with CMV and is a potent activator of bone reabsorption beyond the cells adjacent to it through the co-localization of TACE and RANKL. The intense inflammation seen in the IFM makes it a unique environment where CMV could come out of latency and mediate fibroblast fusion leading to formation of the RPGC.

Confirmation of this hypothesis could lead to future preventative therapies of osteolysis to involve both anti-RANKL treatments and anti-viral treatments.

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